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Full Length Research Paper

Suppression of STAT3 phosphorylation enhances the cytotoxicity of cucurbitacin B in B16F10 melanoma cells

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Cucurbitacin B (CuB) is a triterpenoid compound extracted from Cucurbitaceae plants, which has been shown to induce the phosphorylation (activation) of signal transducer and activator of transcription 3 (STAT3) in melanoma cells. In this study, we aimed to investigate whether inhibition of STAT3 phosphorylation could influence the cytotoxicity of CuB in melanoma cells. The results showed that pretreatment of B16F10 cells with AG490, an inhibitor of Janus kinase 2 (Jak2), enhanced the inhibitory effects of CuB on cell growth and G_2/M phase arrest. DNA content analysis demonstrated that combined AG490 and CuB treatment increased the proportion of apoptotic cells, but decreased the ratio of tetraploid cells. Western blot analysis revealed that AG490 partially suppressed the levels of phosphorylated STAT3 (p-STAT3) and p38 (p-p38) in CuB-treated cells. Moreover, CuB-induced STAT3 phosphorylation could be completely blocked by SP600125, a specific inhibitor of c-Jun N-terminal kinase (JNK) signaling. In addition, SP600125 could also significantly augment CuB-induced apoptotic cell death. These results demonstrated that CuB-induced STAT3 activation was mediated by both JNK and Jak2 signaling and blocking STAT3 activation enhanced the cytotoxicity of CuB in B16F10 melanoma cells.

Key words: AG490, cucurbitacin B, B16F10 melanoma cells, signal transducer and activator of transcription 3 (STAT3), c-Jun N-terminal kinase (JNK).

INTRODUCTION

Cucurbitacin B (CuB), a member of the cucurbitacins (highly oxygenated tetracyclic triterpenes), is extracted from *Trichosanthes kirilowii Maximowicz* (*Cucurbitaceae* family), a plant that has long been used in oriental medicines for its anti-inflammatory, anti-diabetic, and abortificient effects (Blaskovich et al., 2003; Duncan et al., 1996; Jayaprakasam et al., 2003; Oh et al., 2002; Peters et al., 1997, 2003; Yesilada et al., 1988). Recently, accumulating evidence has revealed that CuB and its relatives inhibit the proliferation of various human cancer cells both *in vitro* and in xenografted tumor models, including breast cancer, glioblastoma multiforme, myeloid leukemia, pancreatic cancer, laryngeal cancer, hepatocellular carcinoma cells, and melanoma cells (Blaskovich et al., 2003; Haritunians et al., 2008; Liu et al., 2008; Oh et al., 2002; Thoennissen et al., 2009; Wakimoto et al., 2008; Yin et al., 2008; Chan et al., 2010; Zhang et al., 2011).

Signal transducer and activator of transcription 3 (STAT3) is an oncogene that is aberrantly activated in many types of human cancers, including hepatomas, lymphomas, multiple myelomas and cancers of prostate, breast, lung, head and neck, ovary and stomach in origin (Costantino and Barlocco, 2008; Rigourd et al., 2008). As a member of transcription factor family, STAT3 has been implicated in malignant transformation and tumor cell survival, and therefore has been proposed as target for

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anticancer therapy (Al Zaid Siddiquee and Turkson, 2008; Bowman et al., 1999; Sun et al., 2008). CuB has been found to inhibit the activation of STAT3 (Yin et al., 2008; Zhang et al., 2009). Furthermore, several studies showed that CuB can induce cell apoptosis in a variety of human malignant cell lines and xenografted tumor models by inhibiting Jak2/STAT3 pathway (Haritunians et al., 2008; Wakimoto et al., 2008; Yin et al., 2008; Chan et al., 2010). However, subsequent studies indicated that the inhibition of STAT3 by CuB is cell type-dependent and the anti-proliferative effect of cucurbitacins is independent of STAT3 pathway (Chan et al., 2010; Yasuda et al., 2010; Zhang et al., 2010). Interestingly, our previous report showed that CuB even activates STAT3 in murine melanoma cells (Ouyang et al., 2011). It is still unknown what the underlying mechanism is and whether the STAT3 activation has contributed to the cytotoxicity of CuB on melanoma cells.

Previous results have revealed that CuB can inhibit the growth of B16F10 melanoma cells both *in vitro* and *in vivo* (Ouyang et al., 2011; Zhang et al., 2011). However, CuB can induce the phosphorylation of STAT3 and c-Jun N-terminal kinase (JNK), while the apoptosis of the melanoma cells is relatively low (Ouyang et al., 2011). The purpose of this study was to further investigate the relationship between STAT3 phosphorylation and antitumor effect of CuB in B16F10 melanoma cells. We analyzed the effects of a specific Jak2/STAT3 inhibitor, AG490, on cell growth, cell-cycle distribution, apoptosis, and the expression of proteins involved in the regulation of cell cycle and apoptosis pathway. The results indicated that CuB-induced STAT3 activation was mediated by both JNK and Jak2 signaling, while blocking STAT3 phosphorylation could enhance the cytotoxicity of CuB in B16F10 melanoma cells.

MATERIALS AND METHODS

Chemical reagents

AG490 was purchased from Calbiochem (San Diego, CA, USA), dissolved in dimethyl sulfoxide (DMSO) at 100 mM and stored at −20°C. Cucurbitacins B (CuB; molecular weight 558.7; 98%) was obtained from Zhongxin Innova Laboratories (Tianjin, China), dissolved in dimethylsulphoxide (DMSO) at 20 mM and stored at −20°C. A diluted working solution was prepared freshly before each experiment. DMSO and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). RPMI-1640, fetal bovine serum (FBS) and RNase A were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). The antibodies against phospho-STAT3 (Y705) (#9145), phospho-p38 (#4511), p38 (#9212), JNK (#9258), p-JNK (#4668) and β-tubulin (#2128) were products of Cell Signaling Technologies (Danvers, MA, USA), while the antibody STAT3 (sc-7179) was bought from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The other antibodies, goat anti-mouse IgG HRP conjugate and goat anti-rabbit IgG HRP conjugate were from Jackson ImmuneResearch (West Grove, PA, USA). CellTiter 96 Aqueous ONE Solution kit (MTS) was purchased from Promega (Madison, WI, USA).

Cell culture

Mouse B16F10 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), cultured in Dulbecco's Modified Eagle's Medium (DMEM; high glucose) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin (Invitrogen) and maintained at 37°C in a humidified incubator of 5% CO_2.

Cell proliferation and viability measurement

Cell viability was measured by MTS assay. Briefly, cells were seeded into 96-well plates at 5×10^3 cells per well (100 μL) for 24 h. The next day, the medium was replaced with fresh medium containing appropriate concentrations of drugs or vehicle (0.1% DMSO). After incubation for additional 24 or 48 h, 20 μL MTS reagent was added to each well and incubated at 37°C for 1 to 4 h. The absorbance at 490 nm was measured using a microplate reader (Model 680; Bio-Rad, Richmond, USA). Three independent experiments were performed, each in triplicates. The 50% inhibition concentration (IC_{50}) was derived from the dose-response curve.

Cell morphology

Cell morphology was monitored under a light microscope. In total, 2×10^4 cells were seeded in 24-well plates and allowed to attach overnight. Thereafter, the cells were treated with appropriate concentrations of drugs or vehicle (0.1% DMSO) for 24 h. Cell morphology was photographed (10× magnifications).

Cell cycle analysis

Analysis of cell cycle was performed as described previously (Ouyang et al., 2011). In brief, cells were fixed with 70% ethanol and stained with phosphate-buffered saline (PBS) containing 50 μg/ml propidium iodide (PI) and 30 μg/ml of RNase A. DNA content data were acquired using CELLQuest software on a flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, CA , USA).

Western blot analysis

Western blotting was performed essentially as described previously (Ouyang et al., 2011). In brief, cell lysates were prepared by lysing PBS-washed cells with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Haimen, China). Protein concentration was determined by bicinchoninic acid (BCA) reagents (Pierce; Rockford, IL, USA). Forty micrograms of total protein was separated by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) followed by electro-transfer to polyvinylidene difluoride membrane (Hybond-P; GE Healthcare Life Sciences, USA). The blots were probed with indicated antibodies and then visualized by enhanced chemiluminescence kit (BeyoECL Plus; Beyotime) and recorded on X-ray films (Kodak; Xiamen, China). The densitometry of each band was quantified by FluorChem 8000 (AlphaInnotech; San Leandro, CA, USA).

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA followed by Newman-Keuls multiple comparison test was used to analyze the statistical significance among three or more groups. *P* values < 0.05 are

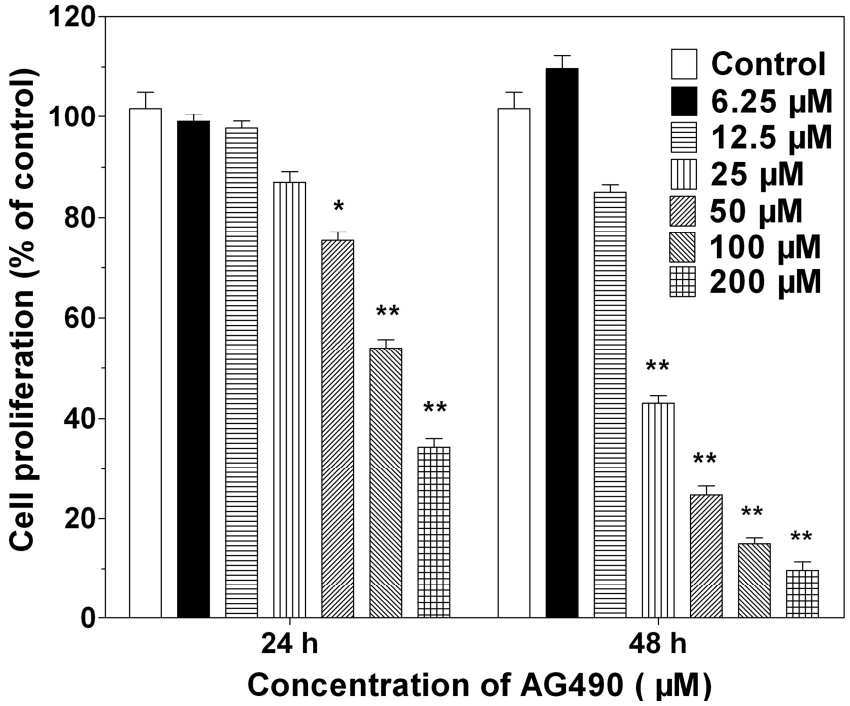

Figure 1. AG490 showed dose- and time-dependent cytotoxicity on mouse B16F10 melanoma cells. B16F10 cells were treated with indicated concentrations of AG490 and cell viability was evaluated by MTS assay. Three independent experiments were performed, each in triplicates. Data are presented as mean ± SD (n = 3). *P < 0.05 and **P < 0.01.

considered statistically significant.

RESULTS

Evaluation of AG490′s cytotoxicity on melanoma cells

As shown in Figure 1, treatment with AG490 showed dose-dependent cytotoxicity on B16F10 cells. After treatment with 12.5 μM AG490 for 24 and 48 h, the proliferation rate of B16F10 melanoma cells was decreased to 96.07 and 83.60%, respectively. When the concentration of AG490 was increased to 25 μM, the proliferation rate was decreased to 85.57 and 42.29%, respectively. Since AG490 showed low cytotoxicity on B16F10 cells when the incubation dose and time were limited to 25 μM and 24 h respectively, we chose 20 μM as the working concentration of AG490 for further study.

Effect of AG490 on the cell proliferation of CuB-treated B16F10 cells

Consistent with our previous study, CuB inhibited the proliferation of B16F10 cells in a time- and dose-dependent manner (Figure 2A and B). Although single agent of 20 μM AG490 showed a relatively low cytotoxicity on B16F10 cells, the proliferation rates of CuB-treated cells were further decreased when these cells had been pretreated with 20 μM AG490 for 2 h, indicating that AG490 could enhance the growth-inhibitory effect of CuB on B16F10 cells.

Effect of AG490 on the morphology of CuB-treated B16F10 cells

The cells treated with single-agent AG490 displayed normal morphology when observed under a light microscope (Figure 3A and B). Treatment of cells with low dose of CuB (0.1 μM) for 24 h did not significantly change the cell morphology, but when the doses of CuB increased to 1 and 10 μM, the cells began to shrink and then became round or even detached from the flasks. Pretreatment of the cells with AG490 had little effect on CuB-induced morphologic changes except that the volume of those cells that treated with combined AG490 and low dose CuB (0.1 μM) was slightly enlarged.

AG490 pretreatment changed the cell cycle distribution of CuB-treated B16F10 cells

Previously, we reported that CuB induced G_2/M phase

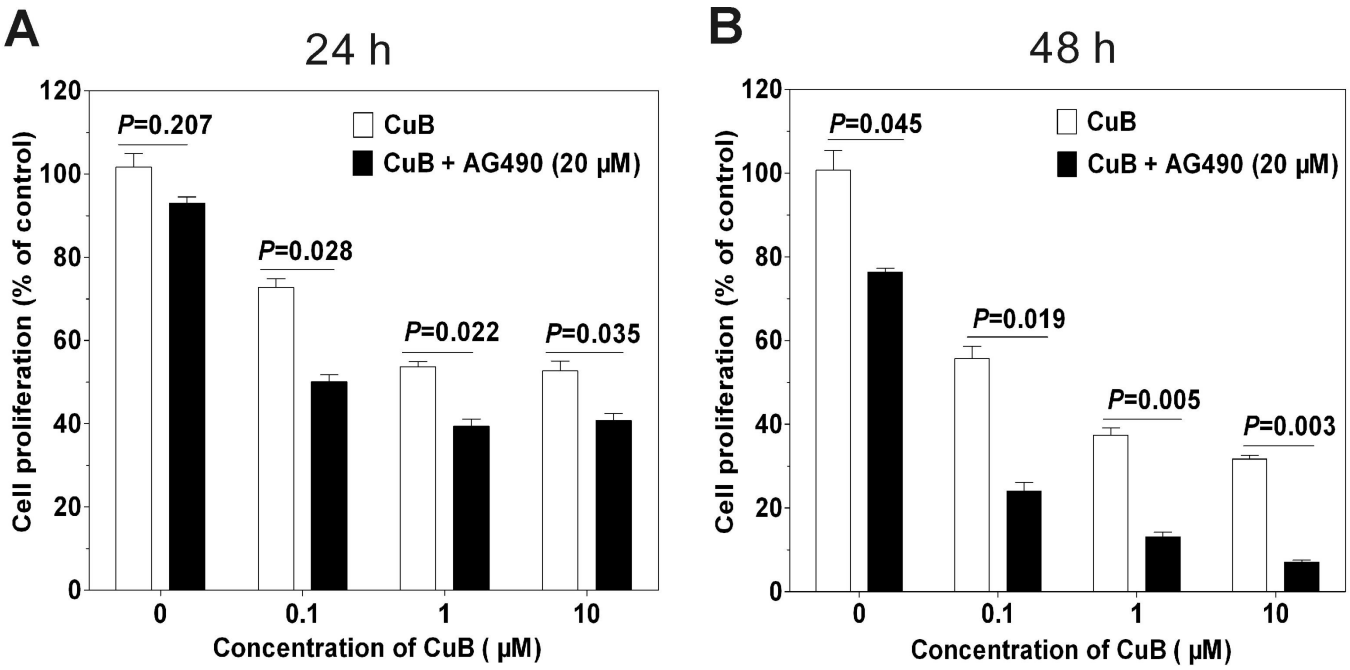

Figure 2. AG490 enhanced the inhibitory effect of CuB on the proliferation of mouse B16F10 melanoma cells. Cells were pretreated with 20 μM AG490 for 2 h and then co-cultured with or without CuB for additional 24 h (A) and 48 h (B). Cell proliferation was assayed by MTS assay. Three independent experiments were performed, each in triplicates. Data are presented as mean ± SD (n=3).

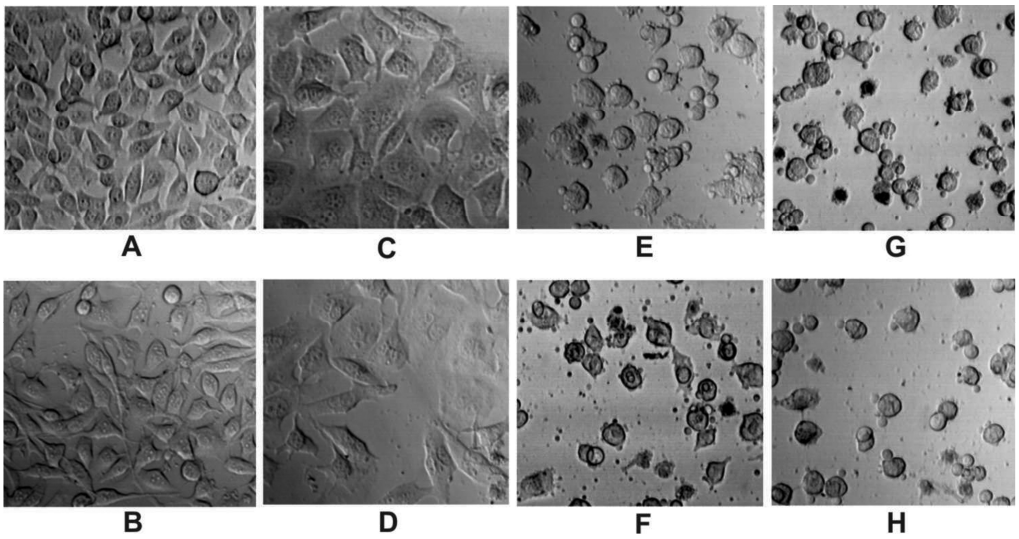

Figure 3. Effect of AG490 on the cell morphology of mouse B16F10 melanoma cells treated with CuB. Cells were pretreated with 20 μM AG490 for 2 h, and then co-cultured with or without CuB for additional 24 h. Phase-contrast images were collected under a light microscope. A, Control; B, AG490; C, CuB (0.1 μM); D, CuB (0.1 μM) + AG490; E, CuB (1 μM); F, CuB (1 μM) + AG490; G, CuB (10 μM); H, CuB (10 μM) + AG490.

arrest and multi-nucleation in B16F10 cells (Zhang et al., 2011). In this study, we evaluated whether pretreatment of the cells with AG490 changed the effect of CuB on cell cycle distribution. The result showed that the groups treated with combined AG490 and CuB had greater proportions of G_2/M phase cells as compared with those treated with CuB alone (Figure 4). Combination of AG490 and CuB also increased the proportions of sub-G_0/G_1 cells as compared to the single agent treatments. However, the percentages of CuB-induced tetraploid cells were significantly decreased by AG490 pretreatment. These results demonstrated that AG490 pretreatment,

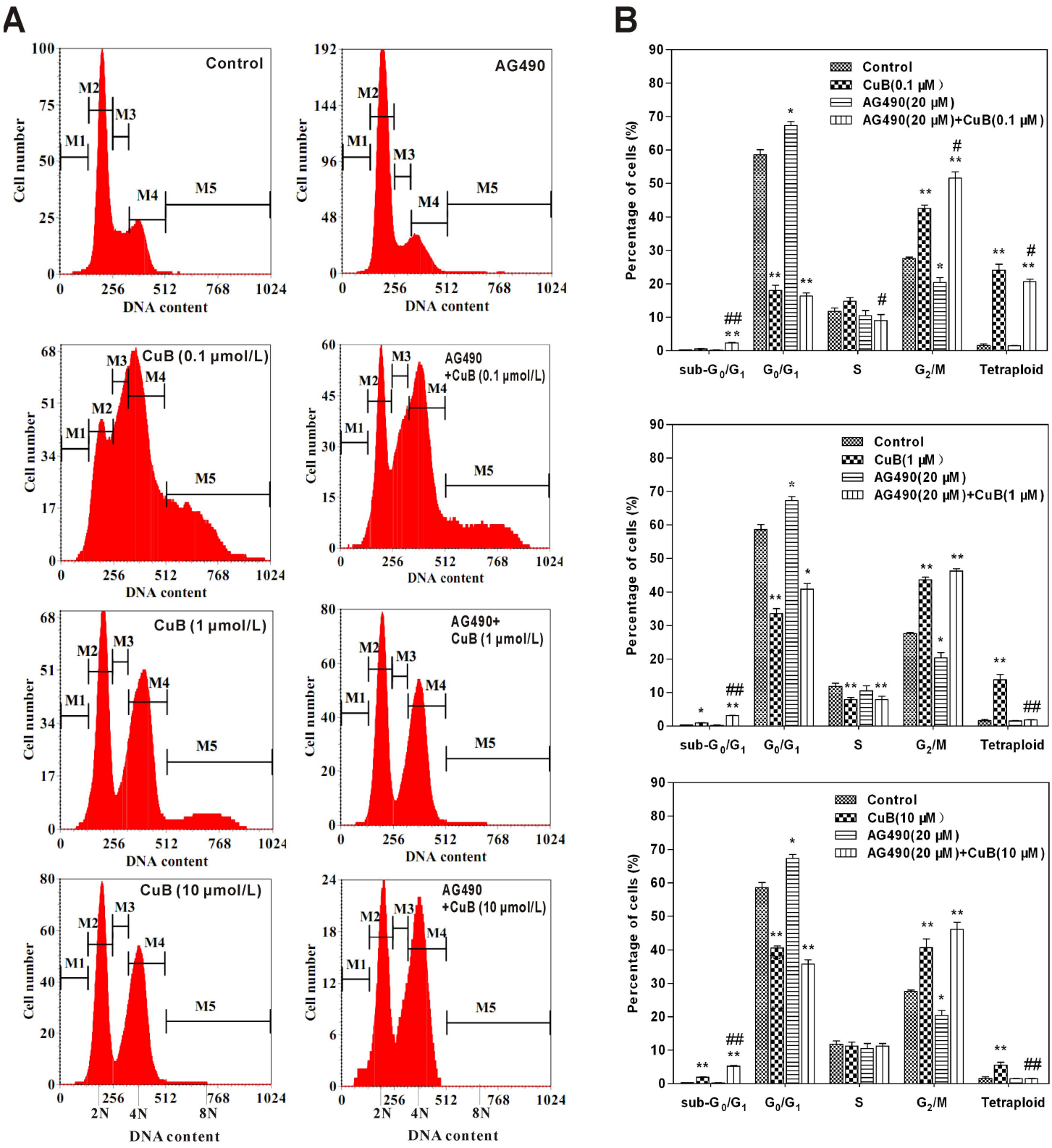

Figure 4. Effect of AG490 on the cell cycle distribution and DNA fragmentation of B16F10 cells treated with CuB for 24 h. After treatment, cells were stained with propidium iodide (PI) and DNA contents were evaluated by flow cytometry. Both flow cytometric data (A) and histogram (B) are presented. Data in the histograms are mean ± SD (n =3) from one of the three independent experiments. The 2N, 4N and 8N indicate cells with diploid, tetraploid (or binuclear) and 8-ploid (or 4 nuclear) DNA contents, respectively. M1, Sub-G_1/G_0 phase; M2, G_1/G_0 phase; M3, S phase; M4, G_2/M phase; M5, tetraploid cells. * $P < 0.05$ and ** $P < 0.01$ vs. control; # $P < 0.05$ and ## $P < 0.01$ vs. CuB group (CuB 0.1 μM + AG490 vs. CuB 0.1 μM, CuB 1 μM + AG490 vs. CuB 1 μM, CuB 10 μM + AG490 vs. CuB 10 μM).

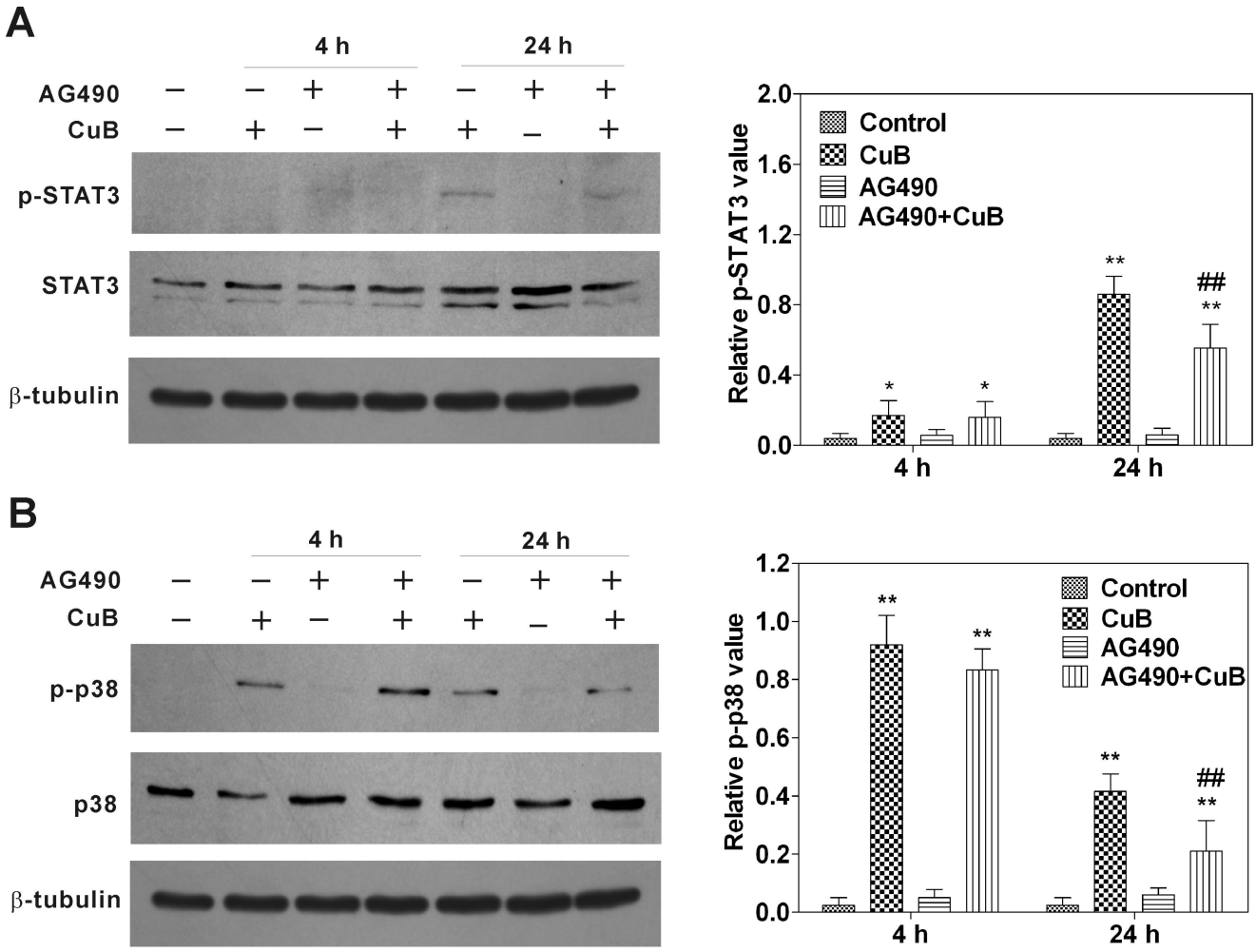

Figure 5. Expression of p-STAT3 and p-p38 in CuB-treated B16F10 cells pretreated with AG490. Cells were pretreated with 20 μM AG490 for 2 h and then co-cultured with or without CuB for additional 4 and 24 h. Cell lysates were resolved by electrophoresis, transferred to poly(vinylidene fluoride) (PVDF) and probed with indicated antibodies. One representative of three independent experiments is presented. The relative densitometry values of each band were normalized to total STAT3 (A), total p38 (B). * $P < 0.05$ and ** $P < 0.01$ vs. control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. CuB group.

which changed the cell cycle distribution of CuB-treated B16F10 cells, enhanced the cytotoxicity of CuB.

STAT3 phosphorylation was mediated by Jak2 and JNK pathways in CuB-treated B16F10 cells

STAT3 signaling plays an important role in cell growth, proliferation and survival (Al Zaid Siddiquee and Turkson, 2008; Battle and Frank, 2002; Costantino and Barlocco, 2008). Previously, we have demonstrated that CuB could induce STAT3 phosphorylation (activation) in B16F10 melanoma cells (Ouyang et al., 2011). To examine the effect of AG490 on STAT3 phosphorylation in CuB-treated cells, the B16F10 melanoma cells were exposed for 2 h with AG490 (20 μM) before the addition of CuB. Western blot analysis showed that CuB treatment time-dependently activated STAT3, while AG490 pretreatment partially suppressed STAT3 activation (Figure 5A). Furthermore, we next examined if mitogen-activated protein kinase (MAPK) pathways were activated by AG490 or their combination in CuB-treated B16F10 cells. Western blot analysis showed that p38 was also activated in CuB-treated cells (Figure 5B). However, pretreatment of the cells with AG490 seemed to have little influence on the CuB-induced p38 activation.

Consistent with previous reports (Zhang et al. 2011), we also found that JNK was rapidly activated by CuB treatment, which could be blocked by the JNK-specific inhibitor SP600125 (Figure 6A). Although SP600125 itself had no effect on STAT3 phosphorylation, treatment of the cells with this compound completely blocked CuB-induced activation of STAT3. This result indicated that CuB-induced STAT3 phosphorylation might also be

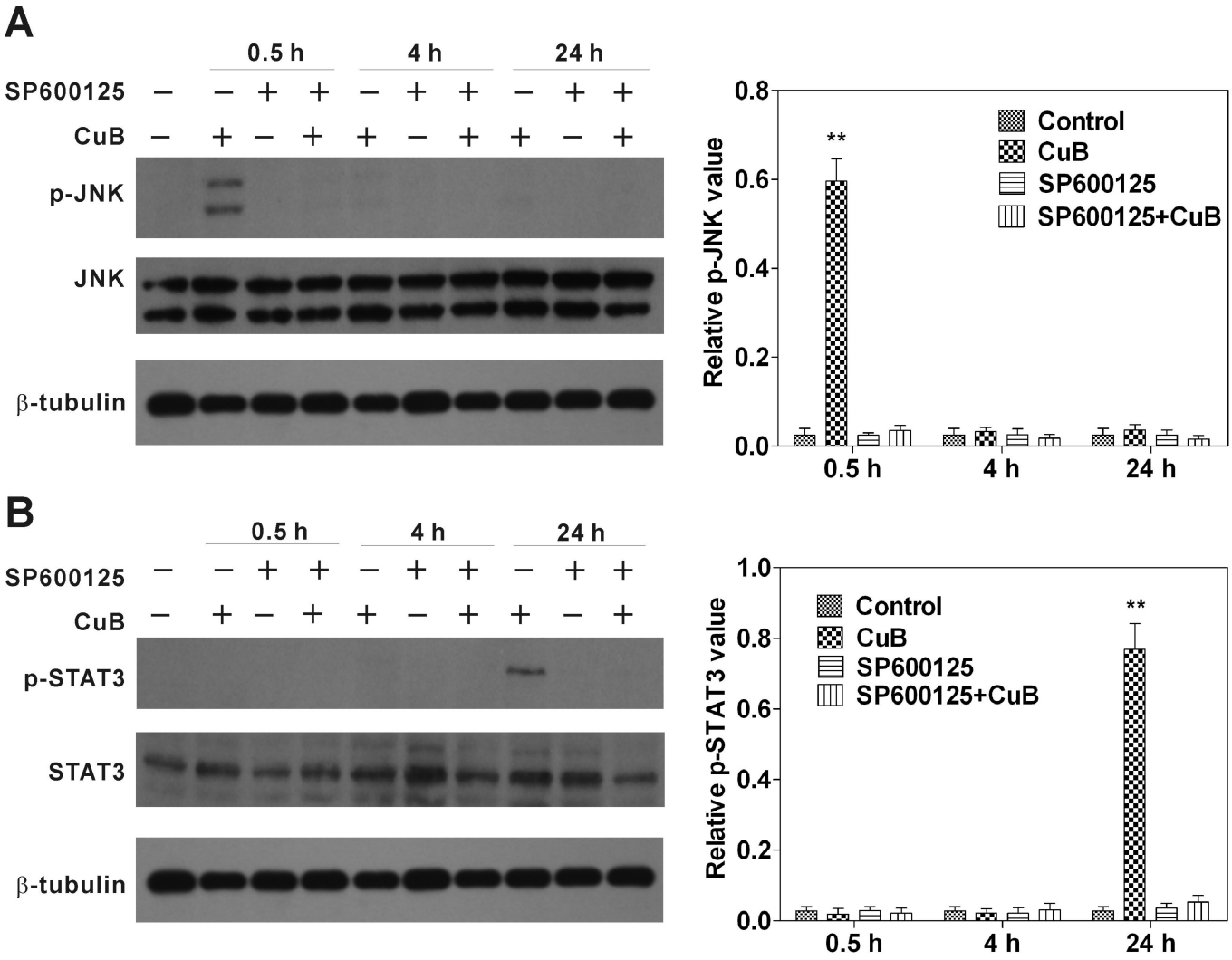

Figure 6. Effect of JNK pathway inhibitor SP600125 on the activation of STAT3 induced by CuB treatment. Cells were pretreated with 40 μM SP600125 for 2 h and then co-cultured with or without CuB for additional 0.5, 4 and 24 h. Cell lysates were resolved by electrophoresis, transferred to PVDF and probed with indicated antibodies. One representative of three independent experiments is presented. The relative densitometry value of each band is normalized to total JNK (A), total STAT3 (B). $^{**}P < 0.01$ vs. control group.

mediated by JNK signaling.

SP600125 pretreatment augmented CuB-induced apoptotic cell death in B16F10 cells

Next, we evaluated the apoptotic effect of SP600125 on CuB-treated melanoma cells with propidium iodide staining. As shown in Figure 7, the percentage of cells in sub-G_0/G_1 phase, which represents apoptosis, was increased from 0.83 ± 0.13% in CuB-treated cells to 34.15±2.15% (1 μM CuB and SP600125) and 26.72±1.73% (10 μM CuB and SP600125) in cells with combined treatment, respectively. These results indicated that pretreatment with SP600125 could augmented apoptotic cell death in CuB-treated B16F10 cells.

DISCUSSION

In this study, we investigated whether suppression of STAT3 activation could influence the cytotoxicity of CuB in B16F10 melanoma cells. We found that Jak2/STAT3 inhibitor (AG490) partially downregulated CuB-induced phosphorylation of STAT3, decreased the formation of tetraploid cells, and increased apoptotic cell death in CuB-treated B16F10 cells, indicating that AG490 could enhance the inhibitory effects of CuB on the cells. These results suggest that CuB-induced phosphorylation of STAT3 may be a compensatory response and suppression of this compensatory response seems to overcome resistance of cells to CuB treatment.

STAT3 activation (phosphorylation) is believed to be regulated by Jak2 that is activated through membrane-

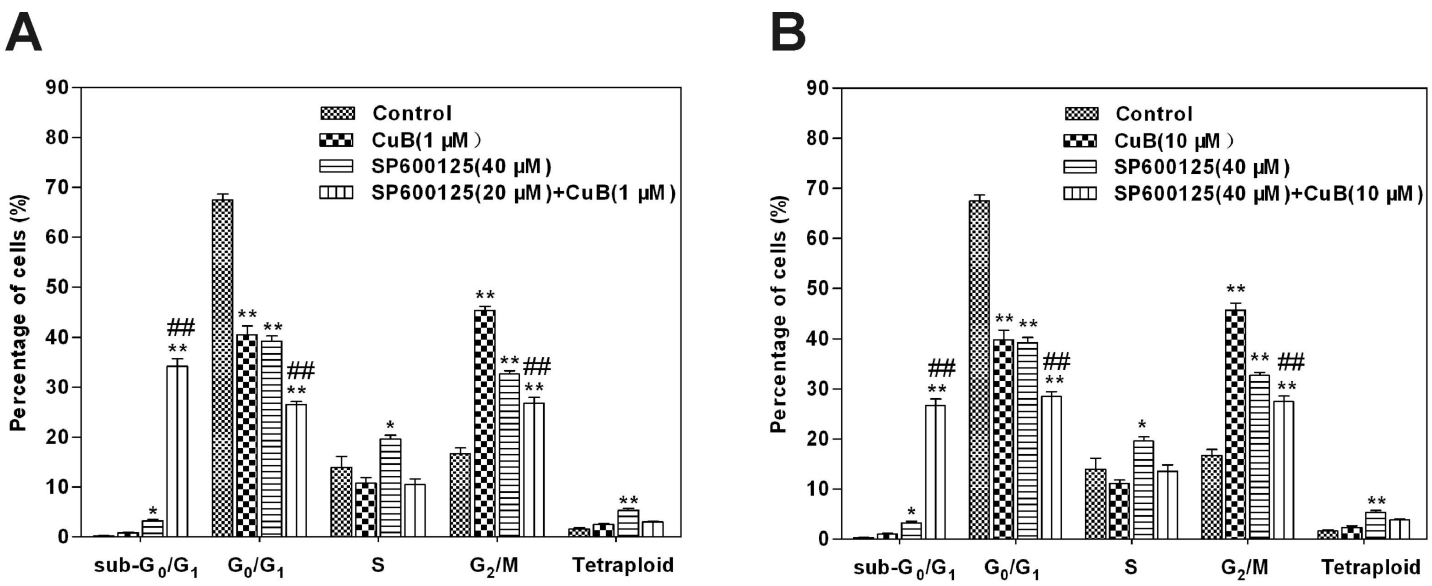

Figure 7. Effect of SP600125 on apoptosis and cell cycle distribution of B16F10 cells treated with CuB for 24 h. Histograms are presented and data in the histograms are mean ± SD (n = 3) from one of three independent experiments. A, Cells were pretreated with 40 μM SP600125 for 2 h and then co-cultured with or without CuB (1 μM) for additional 24 h. B, Cells were pretreated with 40 μM SP600125 for 2 h and then co-cultured with or without CuB (10 μM) for additional 24 h. * $P < 0.05$ and ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. CuB group (CuB 1 μM + SP600125 vs. CuB 1 μM, CuB 10 μM + SP600125 vs. CuB10 μM).

bound receptor (Bowman et al., 2000; Ram and Waxman, 1997). By using Jak2/STAT3 inhibitor AG490, we have demonstrated that AG490 could only partially reduce the levels of CuB-induced phosphorylation of STAT3 in B16F10 cells, which indicated that the stimulation of STAT3 signaling pathway may be also regulated by other kinases. Interestingly, ample evidence has shown that STAT proteins may serve as a natural substrate of mitogen-activated protein kinases (MAPKs), and some stimuli could simultaneously elicit stimulation of STAT3 and MAPK signaling pathways, indicating that activation of MAPK may be associated with stimulation of STAT3 signaling pathway (Decker and Kovarik, 1999; Kovarik et al., 2001; Stephanou et al., 2001). Likewise, the MAPK pathways may also mediate a large variety of external signals, leading to a wide range of cellular responses including growth, differentiation, inflammation and apoptosis (Kyriakis and Avruch, 2001).

Stephanou et al. (2001) found that p38 MAPK is able to phosphorylate Ser-727 of STAT1 and STAT3 *in vitro* directly, and p38-dependent STAT1 and STAT3 Ser-727 phosphorylation has been observed in response to cellular activation in various experimental settings, providing a molecular basis for cross-talk between the p38 MAPK and STAT signaling pathways (Decker and Kovarik, 1999; Gollob et al., 1999; Stephanou et al., 2001). Although we currently have not analyzed STAT3 phosphorylation at Ser-727, our results demonstrated that CuB treatment induced not only STAT3 phosphorylation at Tyr705 but also p38 phosphorylation, and the levels of CuB-induced activated p38 could also be slightly reduced by AG490, indicating that blockage of Jak2/STAT3 pathway may affect the activation of MAPK including p38. Moreover, our previous results indicated that CuB also induces JNK phosphorylation in B16F10 melanoma cells (Ouyang et al., 2011). JNK is a serine/ threonine protein kinase that phosphorylates c-Jun, a component of the activator protein-1 transcription factor complex. In the present study, we found that CuB-induced STAT3 activation was totally blocked by JNK-specific inhibitor SP600125 in B16F10 cells, indicating that CuB-induced JNK activation may be involved in STAT3 phosphorylation. Taken together, our results suggest that the CuB-induced STAT3 phosphorylation is mediated by JNK signaling and to a less extent by Jak2 activity.

Although several studies have suggested that cucurbitacins exert anticancer activities against several human cancer cell lines and tumor xenografts via suppression of STAT3 phosphorylation (Blaskovich et al., 2003; Liu et al., 2008; Shi et al., 2006; Sun et al., 2005; Thoennissen et al., 2009; Zhang et al., 2009), accumulating evidence indicates that disruption of actin cytoskeleton has been involved in the anticancer effect of these compounds (Duncan et al., 1996; Duncan and Duncan, 1997; Yin et al., 2008). They induce quick clumping of F-actin, rapid morphology alteration and actin aggregates in glioblastoma multiforme, breast cancer and myeloid leukemia cells (Haritunians et al., 2008; Wakimoto et al., 2008; Yin et al., 2008). Furthermore, cucurbitacin-induced disturbance of actin dynamics may at least partially account for its inhibitory activities on cell cycle arrest, proliferation, formation of tetraploid cells and migration of

cancer cells (Zhang et al., 2011). Consistent with these studies, our data indicate that treatment with CuB could induce not only G_2/M-phase arrest, but also formation of tetraploid cells. Notably, treatment with low concentration CuB (0.1 μM) induced a significant increase in tetraploid cells, whereas treatment with high doses of CuB resulted in lower percentages of tetraploid cells compared with low dose of CuB (0.1 μM) treatment. The underlying reason might be due to the fact that high doses of CuB not only induced G_2/M arrest and tetraploid formation, but also prevented these tetraploid cells from entering into cell division (as revealed by the cells with DNA content ≥ 4 N). Although the exact mechanism of CuB on actin cytoskeleton has not been identified, it is probably that CuB-induced disruption of actin cytoskeleton confers substantial stress on cells leading to the activation of JNK, which is also known as stress-activated protein kinase (SAPK). Thus, STAT3 phosphorylation may be a downstream effect of JNK and Jak2 signaling accompanied with CuB-induced actin cytoskeleton disruption in murine melanoma cells.

In summary, our results suggest that CuB-induced activation of STAT3 is a compensatory response which may be mediated by both JNK and Jak2 signaling. Suppression of STAT3 activation could enhance the cytotoxicity of CuB in B16F10 melanoma cells, suggesting that STAT3 activation may render the cells resistance to the CuB's anticancer activity.

ACKNOWLEDGEMENTS

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REFERENCES

Al Zaid Siddiquee K, Turkson J (2008). STAT3 as a target for inducing apoptosis in solid and hematological tumors. Cell Res., 18: 254-267.

Battle TE, Frank DA (2002). The role of STATs in apoptosis. Curr. Mol. Med., 2: 381-392.

Blaskovich MA, Sun J, Cantor A, Turkson J, Jove R, Sebti SM (2003). Discovery of JSI-124 (cucurbitacin I), a selective Janus kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice. Cancer Res., 63: 1270-1279.

Bowman T, Garcia R, Turkson J, Jove R (2000). STATs in oncogenesis. Oncogene, 19: 2474-2488.

Bowman T, Yu H, Sebti S, Dalton W, Jove R (1999). Signal Transducers and Activators of Transcription: Novel Targets for Anticancer Therapeutics. Cancer Contr., 6: 427-435.

Chan KT, Meng FY, Li Q, Ho CY, Lam TS, To Y, Lee WH, Li M, Chu KH, Toh M (2010). Cucurbitacin B induces apoptosis and S phase cell cycle arrest in BEL-7402 human hepatocellular carcinoma cells and is effective via oral administration. Cancer Lett., 294: 118-124.

Chan KT, Li K, Liu SL, Chu KH, Toh M, Xie WD (2010). Cucurbitacin B inhibits STAT3 and the Raf/MEK/ERK pathway in leukemia cell line K562. Cancer Lett., 289: 46-52.

Costantino L, Barlocco D (2008). STAT 3 as a target for cancer drug discovery. Curr. Med. Chem., 15: 834-843.

Decker T, Kovarik P (1999). Transcription factor activity of STAT proteins: structural requirements and regulation by phosphorylation and interacting proteins. Cell. Mol. Life Sci., 55: 1535-1546.

Duncan KL, Duncan MD, Alley MC, Sausville EA (1996). Cucurbitacin E-induced disruption of the actin and vimentin cytoskeleton in prostate carcinoma cells. Biochem. Pharmacol., 52: 1553-1560.

Duncan MD, Duncan KL (1997). Cucurbitacin E targets proliferating endothelia. J. Surg. Res., 69: 55-60.

Gollob JA, Schnipper CP, Murphy EA, Ritz J, Frank DA (1999). The functional synergy between IL-12 and IL-2 involves p38 mitogen-activated protein kinase and is associated with the augmentation of STAT serine phosphorylation. J. Immunol., 162: 4472-4481.

Haritunians T, Gueller S, Zhang L, Badr R, Yin D, Xing H, Fung MC, Koeffler HP (2008). Cucurbitacin B induces differentiation, cell cycle arrest, and actin cytoskeletal alterations in myeloid leukemia cells. Leuk. Res., 32: 1366-1373.

Jayaprakasam B, Seeram NP, Nair MG (2003). Anticancer and anti-inflammatory activities of cucurbitacins from *Cucurbita andreana*. Cancer Lett., 189: 11-16.

Kovarik P, Mangold M, Ramsauer K, Heidari H, Steinborn R, Zotter A, Levy DE, Muller M, Decker T (2001). Specificity of signaling by STAT1 depends on SH2 and C-terminal domains that regulate Ser727 phosphorylation, differentially affecting specific target gene expression. EMBO J., 20: 91-100.

Kyriakis JM, Avruch J (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev., 81: 807-869.

Liu T, Zhang M, Zhang H, Sun C, Yang X, Deng Y, Ji W (2008). Combined antitumor activity of cucurbitacin B and docetaxel in laryngeal cancer. Eur. J. Pharmacol., 587: 78-84.

Oh H, Mun YJ, Im SJ, Lee SY, Song HJ, Lee HS, Woo WH (2002). Cucurbitacins from *Trichosanthes kirilowii* as the inhibitory components on tyrosinase activity and melanin synthesis of B16/F10 melanoma cells. Planta Med., 68: 832-833.

Ouyang D, Zhang Y, Xu L, Li J, Zha Q, He X (2011). Histone deacetylase inhibitor valproic acid sensitizes B16F10 melanoma cells to cucurbitacin B treatment. Acta Biochim. Biophys. Sin., 43: 487-495.

Ouyang DY, Ji YH, Saltis M, Xu LH, Zhang YT, Zha QB, Cai JY, He XH (2011). Valproic acid synergistically enhances the cytotoxicity of gossypol in DU145 prostate cancer cells: An iTRTAQ-based quantitative proteomic analysis. J. Proteomics, 74: 2180-2193.

Peters RR, Baier Krepsky P, Siqueira-Junior JM, da Silva Rocha JC, Marques Bezerra M, de Albuquerque Ribeiro R, de Brum-Fernandes AJ, Rocha Farias M, Castro da Rocha FA, Ribeiro-do-Valle RM (2003). Nitric oxide and cyclooxygenase may participate in the analgesic and anti-inflammatory effect of the cucurbitacins fraction from *Wilbrandia ebracteata*. Life Sci., 73: 2185-2197.

Peters RR, Farias MR, Ribeiro-do-Valle RM (1997). Anti-inflammatory and analgesic effects of cucurbitacins from *Wilbrandia ebracteata*. Planta Med., 63: 525-528.

Ram PA, Waxman DJ (1997). Interaction of growth hormone-activated STATs with SH2-containing phosphotyrosine phosphatase SHP-1 and nuclear JAK2 tyrosine kinase. J. Biol. Chem., 272: 17694-17702.

Rigourd V, Chauvet C, Chelbi ST, Rebourcet R, Mondon F, Letourneur F, Mignot TM, Barbaux S, Vaiman D (2008). STOX1 overexpression in choriocarcinoma cells mimics transcriptional alterations observed in preeclamptic placentas. PLoS One, 3: e3905.

Shi X, Franko B, Frantz C, Amin HM, Lai R (2006). JSI-124 (cucurbitacin I) inhibits Janus kinase-3/signal transducer and activator of transcription-3 signalling, downregulates nucleophosmin-anaplastic lymphoma kinase (ALK), and induces apoptosis in ALK-positive anaplastic large cell lymphoma cells. Br. J. Haematol., 135: 26-32.

Stephanou A, Scarabelli TM, Brar BK, Nakanishi Y, Matsumura M, Knight RA, Latchman DS (2001). Induction of apoptosis and Fas receptor/Fas ligand expression by ischemia/reperfusion in cardiac myocytes requires serine 727 of the STAT-1 transcription factor but not tyrosine 701. J. Biol. Chem., 276: 28340-28347.

Sun J, Blaskovich MA, Jove R, Livingston SK, Coppola D, Sebti SM (2005). Cucurbitacin Q: a selective STAT3 activation inhibitor with

potent antitumor activity. Oncogene, 24: 3236-3245.
Sun X, Zhang J, Wang L, Tian Z (2008). Growth inhibition of human hepatocellular carcinoma cells by blocking STAT3 activation with decoy-ODN. Cancer Lett., 262: 201-213.
Thoennissen NH, Iwanski GB, Doan NB, Okamoto R, Lin P, Abbassi S, Song JH, Yin D, Toh M, Xie WD, Said JW, Koeffler HP (2009). Cucurbitacin B induces apoptosis by inhibition of the JAK/STAT pathway and potentiates antiproliferative effects of gemcitabine on pancreatic cancer cells. Cancer Res., 69: 5876-5884.
Wakimoto N, Yin D, O'Kelly J, Haritunians T, Karlan B, Said J, Xing H, Koeffler HP (2008). Cucurbitacin B has a potent antiproliferative effect on breast cancer cells *in vitro* and *in vivo*. Cancer Sci., 99: 1793-1797.
Yasuda S, Yogosawa S, Izutani Y, Nakamura Y, Watanabe H, Sakai T (2010). Cucurbitacin B induces G2 arrest and apoptosis via a reactive oxygen species-dependent mechanism in human colon adenocarcinoma SW480 cells. Mol. Nutr. Food Res., 54: 559-565.
Yesilada E, Tanaka S, Sezik E, Tabata M (1988). Isolation of an anti-inflammatory principle from the fruit juice of *Ecballium elaterium*. J. Nat. Prod., 51: 504-508.
Yin D, Wakimoto N, Xing H, Lu D, Huynh T, Wang X, Black KL, Koeffler HP (2008). Cucurbitacin B markedly inhibits growth and rapidly affects the cytoskeleton in glioblastoma multiforme. Int. J. Cancer, 123: 1364-1375.
Zhang M, Sun C, Shan X, Yang X, Li-Ling J, Deng Y (2010). Inhibition of pancreatic cancer cell growth by cucurbitacin B through modulation of signal transducer and activator of transcription 3 signaling. Pancreas, 39: 923-929.
Zhang M, Zhang H, Sun C, Shan X, Yang X, Li-Ling J, Deng Y (2009). Targeted constitutive activation of signal transducer and activator of transcription 3 in human hepatocellular carcinoma cells by cucurbitacin B. Cancer Chemother. Pharmacol., 63: 635-642.
Zhang Y, Ouyang D, Xu L, Ji Y, Zha Q, Cai J, He X (2011). Cucurbitacin B induces rapid depletion of the G-actin pool through reactive oxygen species-dependent actin aggregation in melanoma cells. Acta Biochim. Biophys. Sin., 43: 556-567.