Acta Biochim Biophys Sin 2011, 43: 487–495 | © The Author 2011. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr032.

Original Article

Histone deacetylase inhibitor valproic acid sensitizes B16F10 melanoma cells to cucurbitacin B treatment

Dongyun Ouyang[1†], Yanting Zhang[1†], Lihui Xu[2], Jingjing Li[1], Qingbing Zha[1], and Xianhui He[1*]

[1]Institute of Tissue Transplantation and Immunology, Jinan University, Guangzhou 510632, China
[2]Institute of Bioengineering, Jinan University, Guangzhou 510632, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-20-85220679; Fax: +86-20-85221337; E-mail: thexh@jnu.edu.cn

Cucurbitacin B (CuB) is reported to have anti-proliferation effects on a variety of tumors including melanoma, and more effective regimens by combination of this agent with others are under investigation. In this study, the anti-melanoma effect of CuB as a single agent and in combination with valproic acid (VPA), an inhibitor of histone deacetylase (HDAC), was evaluated in B16F10, a mouse melanoma cell line. The results demonstrated that CuB inhibited the proliferation of the cell line in a dose-dependent manner. However, it was likely that a pro-survival compensatory response, involving the induction of autophagy and upregulation of anti-apoptotic Bcl-2 protein, was induced by CuB treatment, which might greatly decrease the cytotoxicity of this agent. Supporting this, the melanoma cells were found to be more sensitive to the combination of CuB with chloroquine, a well-known autophagy inhibitor. And CuB-induced autophagy was associated with c-Jun N-terminal kinase (JNK) activation, at least partly, since inhibition of JNK activity by SP600125 could alleviate the autophagy. When CuB was combined with VPA, the two drugs showed synergistic cytotoxicity by induction of cell apoptosis. Moreover, the multiploidization effect of CuB was also suppressed in the presence of VPA. In contrast to the transient activation of JNKs by CuB, the combination of CuB and VPA resulted in prolonged JNK activation, although at low level after 4 h. Our results demonstrated that HDAC inhibitor VPA can sensitize B16F10 cells to CuB treatment through induction of apoptotic pathway.

Keywords valproic acid; cucurbitacin B; synergistic effect; melanoma; autophagy; apoptosis

Received: December 29, 2010 Accepted: February 23, 2011

Introduction

Melanoma is the most fatal form of skin cancer with growing incidence throughout the world. There are no efficacious therapies to malignant melanoma by now. The alkylating agent dacarbazine as a single agent remains to be the current standard treatment, although few patients can achieve remission from distant metastases, and the 5-year survival rate is <10% [1]. Thus, new agents and/or therapy strategies with different action targets need to be developed.

Cucurbitacin B (CuB), a triterpenoid compound, is one of the most abundant members of cucurbitacins in *Cucurbitaceae* plants. Cucurbitacins have been used as folk medicines for centuries in China and India for their anti-inflammatory, anti-diabetic, and abortifacient effects [2]. In recent years, a large body of evidence have revealed that CuB and its relatives inhibit the growth of a wide spectrum of human malignant cells both *in vitro* and in xenografted tumors, including breast cancer [3], glioblastoma multiforme [4], myeloid leukemia [5], pancreatic cancer [6], and laryngeal cancer [7]. In order to understand the mechanism of CuB activity on tumor cells, several studies have demonstrated that CuB induces tumor cell apoptosis by downregulation of signal transducers and activators of transcription-3 (STAT3) activation, while it may also exhibit anti-tumor effects without influencing STAT3 activation in human hepatocellular carcinoma (BEL-7402) and colon adenocarcinoma (SW 480) cells [6,8,9]. As a transcription factor, STAT3 regulates a number of genes that are vital for cell proliferation, angiogenesis, and survival. In normal cells, STAT3 is strictly controlled, while in tumor cells, it is often found constitutively activated, which contributes to the malignant transformation and cellular resistance to apoptosis [10–12].

The histone deacetylase (HDAC) inhibitors activate tumor suppressive genes, including p21 and cyclin-dependent kinase inhibitors, and p53, while suppress

oncogene expression by increasing histone acetylation [13–15]. Thus, the combination of conventional chemotherapeutic agents with HDAC inhibitors is believed to be a promising co-therapy for the treatment of drug-refractory tumors by circumventing the activation of survival pathways [16–18]. Valproic acid (VPA), currently used as an anti-convulsant and anti-depressant, has recently garnered attention as an anti-cancer agent. Through specific inhibition of HDAC1 and HDAC2, VPA elongates the G_1 phase of cell cycle [19], and inhibits a myriad of cell growth and replication processes [20]. Recently, VPA has been reported to sensitize small cell lung cancer to the standard chemotherapeutic first-line regimen (cisplatin + etoposide) [17].

In this study, the anti-melanoma effects of CuB as a single agent and in combination with VPA were evaluated in B16F10 mouse melanoma cell line. Our results demonstrated that CuB and VPA independently inhibited the proliferation of B16F10 cells in a dose-dependent manner. As reported in other cancer cells [4–6,8,21], CuB alone inhibited cell proliferation of melanoma cells via G_2/M phase arrest. It also could induce a pro-survival response in melanoma cells, involving induction of autophagy and upregulation of anti-apoptotic Bcl-2 protein. Combination of CuB with VPA showed synergistic cytotoxicity by induction of apoptotic pathway in the cell line. Moreover, the multiploidization effect of CuB was also suppressed in the presence of VPA. Our results suggest that combination of HDAC inhibitors with CuB may provide an effective regimen against melanomas.

Materials and Methods

Chemical reagents

CuB (molecular weight 558.7) with 98% purity was obtained from Zhongxin Innova Laboratories (Tanjin, China), dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored at −20°C. Diluted working solution was prepared freshly before each experiment. Triton X-100, sodium deoxycholate, dithiothreitol, propidium iodide (PI), and DMSO were from Sigma (St Louis, USA). RNase A was purchased from Invitrogen (Carlsbad, USA). The antibodies against phospho-stress-activated protein kinase/c-Jun N-terminal kinase (JNK) (T183/Y185), phospho-STAT3 (Y705), phospho-eIF2α, phospho-H2A.X, acetyl-histone H3K9, histone H3, eIF2α, Bcl-2, BAX, cleaved caspase-3 were products of Cell Signaling Technologies (Danvers, USA). The antibodies against STAT3, survivin, and β-tubulin were bought from Santa Cruz Biotechnology Inc. (Santa Cruz, USA) and anti-LC3B antibody was from Sigma.

Cell culture

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

Measurement of cell proliferation and viability

Cell viability was measured by MTS assay (CellTiter 96 Aqueous ONE Solution kit; Promega, Madison, USA). Briefly, B16F10 cells were seeded into 96-well plates at 5×10^3 cells per well (100 μl) for 24 h. The next day, the medium were replaced with fresh medium containing appropriate concentrations of drugs or vehicle (DMSO). After incubation for 48 h, 20 μl MTS reagent was added to each well and incubated at 37°C for 1–4 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 concentrations (IC_{50}) were derived from the dose–response curve

Cell cycle analysis

B16F10 cells seeded in six well plates were incubated with CuB for 24 or 48 h in the presence of appropriate drugs. Afterwards, the cells in each well were harvested and washed twice with cold phosphate-buffered saline (PBS). Next, the cells were fixed with 70% cold ethanol at −20°C for at least 1.5 h, and then stained with PI solution (50 μg/ml PI with 30 μg/ml RNase A) at 37°C for 1 h. DNA content in each sample was analyzed by a flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, USA).

Western blot analysis

Cells were seeded in 75 cm^2 flasks and treated with indicated drugs or vehicle for 24 h. CuB concentration was 1 μM. Cell lysates were prepared by lysing PBS-washed cells with RIPA buffer (Beyotime, Haimen, China) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH7.4, supplemented with NaF, $NaVO_4$, EDTA, and protease inhibitor cocktail. Supernatants were collected after centrifugation of cell lysates at 12,000 rpm for 30 min at 4°C. Protein concentrations were determined by BCA reagents (Pierce, Rockford, USA). Forty micrograms of total protein was separated by SDS–polyacrylamide gel electrophoresis followed by electro-transfer to polyvinylidene difluoride membrane. For detection of acetylated-histone H3, phospho-H2A.X, H3, and LC3B levels, samples were prepared in 2 × SDS loading buffer followed

by sonication. The blots were probed with indicated antibodies and then visualized by enhanced chemiluminescence (BeyoECL Plus; Beyotime).

Statistical analysis

Data were presented as mean ± SD. Statistical analysis was performed using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, USA). One-way analysis of variance followed by Dunnett's multiple comparison test was used to analyze the statistical significance of three or more groups. *P*-values <0.05 were considered statistically significant.

Results

CuB and VPA synergistically inhibited the proliferation of melanoma cells

As shown in **Fig. 1(A)**, CuB, in a dose-dependent manner, inhibited the proliferation of B16F10 cells with an IC_{50} value of 0.32 ± 0.04 μM [**Fig. 1(A)**]. VPA also showed a dose-dependent inhibition on the cell line. Its IC_{50} value was 11.97 ± 2.42 mM. When the concentration was <1.6 mM, VPA showed little cytotoxicity [**Fig. 1(B)**].

When VPA was combined with CuB, a greater cytotoxicity was detected [**Fig. 1(C)**]. Their combination indexes, as analyzed by the CalcuSyn v2.0 software, were all <0.7, which indicates that the two drugs had strong synergistic activity on inhibition of cell growth [**Fig. 1(D)**].

VPA induced hyperacetylation of histone H3 in melanoma cells

VPA has been discovered not only to inhibit class I HDACs, but also induce proteosomal degradation of class II HDACs [22–24]. In this study, the HDAC inhibitory activity of VPA was evaluated by the hyperacetylation of histone H3 in cells after VPA treatment. The results show that VPA treatment for 24 h significantly upregulated the levels of acetylated histone H3 in melanoma cells, while CuB could slightly reduce the HDAC-inhibiting activity of VPA (**Fig. 2**).

CuB treatment induced endoplasmic reticulum (ER) stress and autophagy in melanoma cells

Since the inhibition of HDAC activity influences the profile of protein expression in cells, we are interested in whether VPA treatment would induce an unfolded protein response (or ER stress). Previous studies have indicated that, on environmental stress, the protein translation initiator eIF2α is phosphorylated by protein kinases and the global protein translation is decreased to alleviate the cellular injury or alternatively induce cell apoptosis [25]. The phosphorylation of eIF2α also mediates the conversion of LC3-I (18 kDa) to LC3-II (16 kDa), an essential step in formation of autophagosome, in response to ER stress [26]. Therefore, ER stress was evaluated by the level of phosphorylated eIF2α in this study. The results showed that a significant upregulation of phosphorylated eIF2α was observed in VPA-treated melanoma cells [**Fig. 3(A)**], suggesting that ER stress was induced by VPA treatment. However, the induction of ER stress by single-agent VPA did not result in induction of autophagy as indicated by levels of LC3-II (16 kDa) [**Fig. 3(B)**].

CuB is reported to inhibit cell proliferation by targeting actin cytoskeleton [4,5]. We also observed that CuB treatment resulted in cell morphology alteration and rapid blebbing in the cells (data not shown), suggesting the disruption of cellular actin cytoskeleton. After treatment with CuB for 24 h, both phosphorylated eIF2α and LC3-II (16 kDa) were markedly upregulated [**Fig. 3(A,B)**], suggesting that CuB could induce both ER stress and autophagy in B16F10 cells, which is similar to other drugs targeting cytoskeleton [27,28]. In addition, autophagy in CuB and VPA combination group was similar to that treated with CuB alone [**Fig. 3(B)**], while the ER stress markers were higher in CuB plus VPA group than in either CuB- or VPA-treated groups [**Fig. 3(A)**].

Suppression of autophagy by chloroquine sensitized melanoma cells to CuB treatment

As shown in **Supplementary Fig. S1**, CuB markedly induced cell cycle arrest in G_2/M phases and formation of multiploid cells. No significant cell apoptosis was observed by the flow cytometry analysis based on PI staining of the DNA contents. These results suggested that the induction of autophagy by CuB might serve as a pro-survival response. In order to confirm this, we determined the cytotoxic and apoptotic effects of CuB plus chloroquine (CQ), a well-known autophagy inhibitor. MTS assay showed that the cell viability was significantly decreased in CuB plus CQ groups in comparison with the CuB-treated group [**Fig. 4(A)**]. Flow cytometry analysis revealed that higher apoptotic peaks were detected in the CuB plus CQ groups as compared with the CuB group [**Fig. 4(B)**]. These results suggested that CuB-induced autophagy in melanoma cells was a pro-survival response and conferred resistance to the cytotoxicity of CuB.

CuB plus VPA led to the prolonged activation of JNK1 and JNK2

Both JNK1 and JNK2 were activated rapidly by CuB, and reached their highest peaks at 30 min, then decreased in a time-dependent manner. The phosphorylated JNKs in CuB-treated cells were decreased back to baseline until 24 h [**Fig. 5(A)**]. The activation of JNKs contributed, at least partly, to CuB-activated autophagy, since the inhibition of JNK by SP600125 lowered the level of LC3-II,

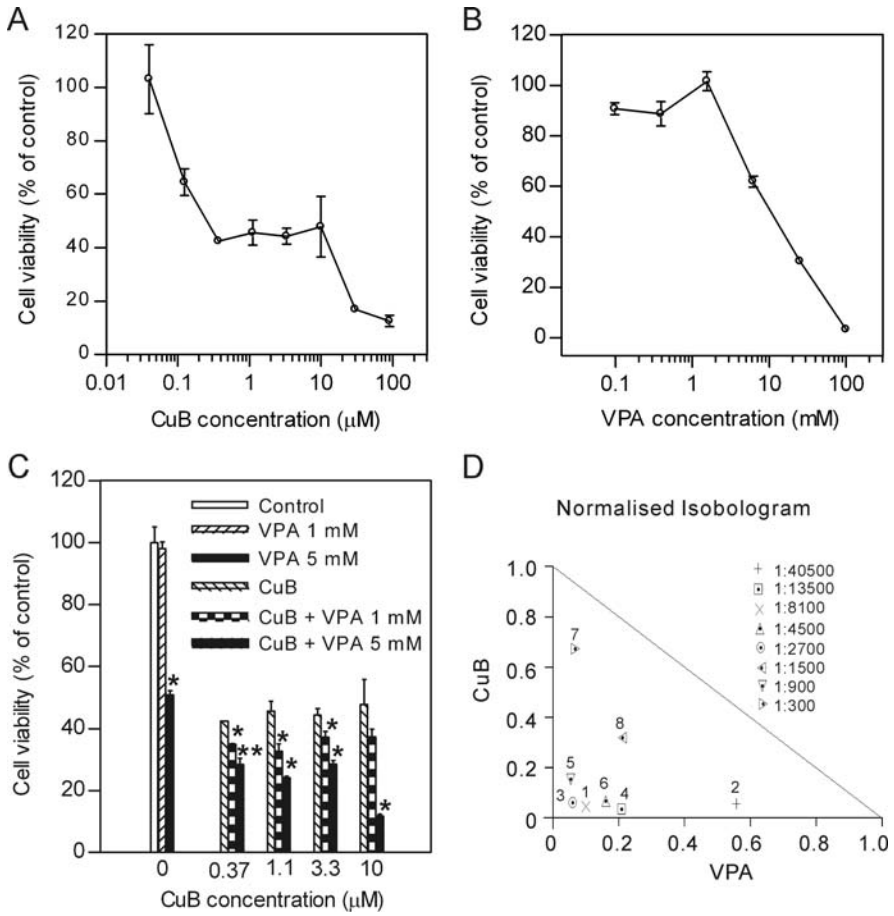

Figure 1 Synergistic inhibition of the proliferation of mouse B16F10 melanoma cells by CuB plus VPA B16F10 cells were treated with CuB and/or VPA for 48 h, and cell viability was evaluated by MTS assay. CuB (A), VPA (B), and their combination (C) in a dose -dependent manner inhibited the growth of B16F10. Data are presented as mean ± SD. ($n = 3$). *$P < 0.05$ and **$P < 0.01$. Drug interaction analysis using the CalcuSyn v2.0 software indicated that CuB and VPA had strong synergistic activity on inhibition of B16F10 cell growth (D).

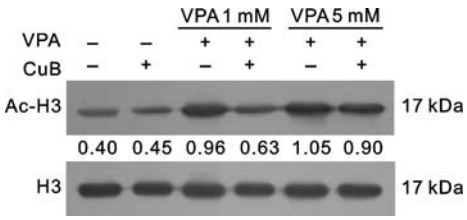

Figure 2 Inhibition of HDAC activity by VPA in melanoma cells B16F10 cells were treated with 1 μM CuB in the presence/absence of VPA for 24 h. Cell lysates were resolved by electrophoresis, transferred to PVDF, and probed with indicated antibodies by western blotting. One representative of three independent experiments is shown. The relative densitometry values of each acetylated histone H3 band was normalized to the total histone H3 levels. Ac-H3, acetylated histone H3.

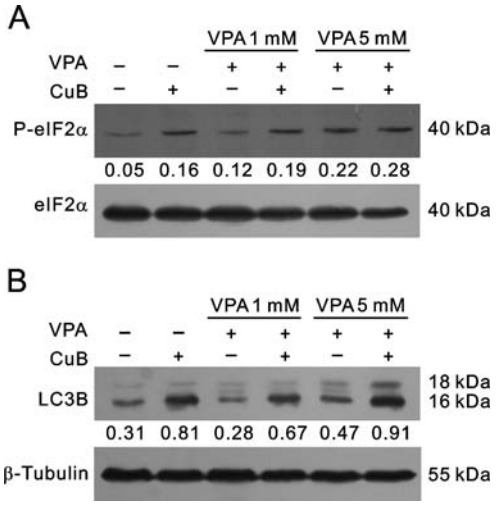

Figure 3 Analysis of ER stress (A) and autophagy (B) induced by CuB and/or VPA treatment B16F10 cells were treated with 1 μM CuB in the presence/absence of VPA for 24 h. Cell lysates were resolved by electrophoresis, transferred to PVDF, and probed with indicated antibodies by western blotting. One representative of three independent experiments is shown. The relative densitometry values of P-eIF2α against eIF2α (A) and LC3-II (16 kDa) against β-tubulin (B) are shown.

an autophagy indicator [**Fig. 5(B)**]. VPA did not induce JNK activation [**Fig. 5(C,D)**], and it also did not induce the significant autophagy [**Fig. 3(B)**]. In contrast to the transient activation of JNKs by CuB, the combination of CuB and VPA resulted in prolonged JNK activation, although at a low level after 4 h [**Fig. 5(C,D)**]. These

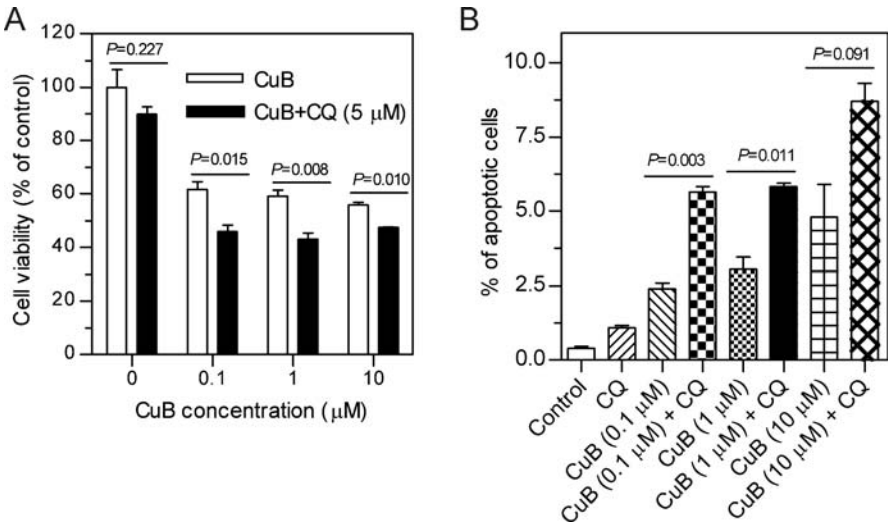

Figure 4 The autophagic pathway inhibitor CQ reduced the cell viability (A) and increased the apoptotic cells (B) of CuB-treated cells Cells were treated with indicated concentration of CuB and 5 μM CQ for 48 h. Cell viability was calculated from MTS assay (A), and the fractions of apoptotic cells were evaluated as the sub-G_0/G_1 peak (apoptotic peak) by flow cytometry (B).

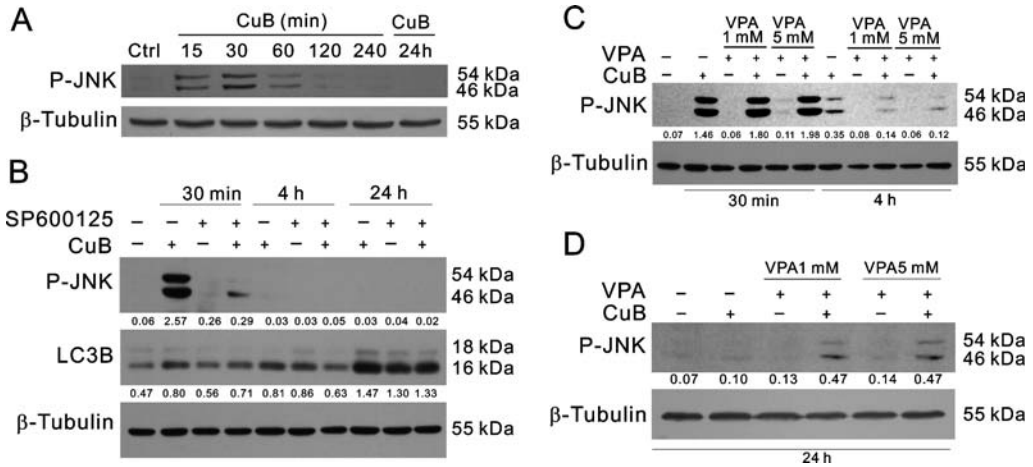

Figure 5 Evaluation of JNK1 and JNK2 activation in melanoma cells (A) B16F10 cells were treated with 1 μM CuB for indicated time. (B) JNK pathway inhibitor SP600125 suppressed the activation of JNKs induced by CuB treatment, and resulted decreased level of LC3-II (16 kDa). (C,D) Combination of CuB and VPA resulted in sustained JNK activation. Cell lysates were resolved by electrophoresis, transferred to PVDF, and probed with indicated antibodies by western blotting. One representative of three independent experiments is shown. The relative densitometry values of each band were normalized to β-tubulin. The values under LC3 were the ratios of LC3-II (16 kDa) against β-tubulin.

results suggested that JNK activation was associated with the induction of autophagy.

CuB and VPA combination resulted in aggravation of DNA damage and cell apoptosis

Next, we evaluated the apoptotic effects of VPA and/or CuB treatments on melanoma cells. One of the typical characteristics of cell apoptosis is DNA fragmentation, which is caused by the activation of caspase-3 and is partly the consequence of failed DNA damage repair. In response to genotoxicity, H2A.X is quickly phosphorylated (resulting in γ-H2A.X) to recruit other repair factors to the damaged site [29,30]. It was observed that treatment with VPA alone increased the level of γ-H2A.X, indicating that VPA was a DNA damage inducer in melanoma cells [**Fig. 6(A)**]. Furthermore, VPA treatment induced cell apoptosis as indicated by cleaved caspase-3 (activation), an executor of the apoptotic pathway [**Fig. 6(B)**]. Flow cytometry analysis also demonstrated that there was a small fraction of VPA-treated cells undergoing DNA fragmentation (sub-G_0/G_1 peak), while a greater fraction was arrested at G_0/G_1 phase [**Fig. 7(A,B)**]. In accordance with the cytotoxicity of VPA [**Fig. 1(B)**], the expression of survivin was also suppressed in a dose-dependent manner by VPA [**Fig. 6(B)**].

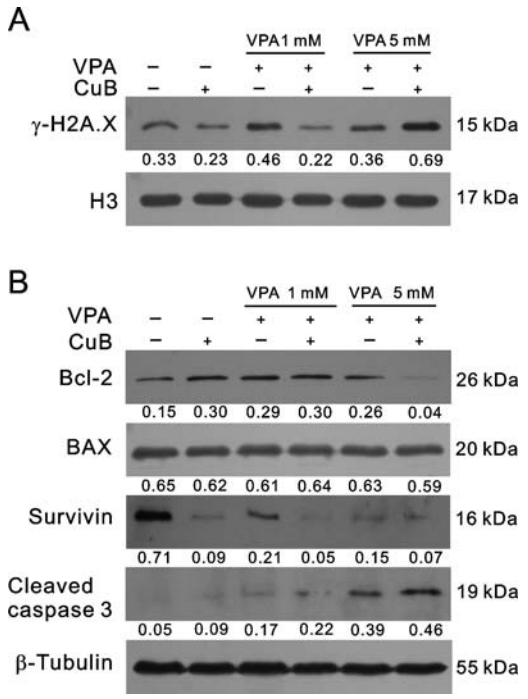

Figure 6 CuB and VPA combination resulted in phosphorylation of H2A.X and activation of apoptosis pathway B16F10 cells were treated with 1 μM CuB and/or VPA for 24 h. Cell lysates were resolved by electrophoresis, transferred to PVDF, and probed with indicated antibodies by western blotting. One representative of three independent experiments is shown. The relative densitometry values of each band were normalized to total histone H3 (A) or β-tubulin (B).

Although 1 μM CuB alone induced a sharp decrease in survivin [**Fig. 6(B)**], which corresponded to the arrest of cell cycle in G_2/M phases [**Fig. 7(A,B)**], it did not activate caspase-3 [**Fig. 6(B)**]. Moreover, CuB treatment resulted in the upregulation of Bcl-2 while BAX expression was not affected. The γ-H2A.X level in CuB-treated cells was even lower than the basal level of control [**Fig. 6(A)**]. Consistent with these results, flow cytometry analysis demonstrated that CuB treatment did not induce a sub-G_0/G_1 peak. Most of the cells were arrested in G_2/M phase and a small fraction of cells were multiploid (>4N) in this group. When incubated with CuB for 48 h, about 22% cells had >4N DNA content [**Fig. 7(A,B)**]. These results suggested that CuB inhibited the proliferation of melanoma cells by arresting cell cycle, while conferred resistance of the cells to apoptosis.

When 1 μM CuB was used with 5 mM VPA for 24 h, both cleaved caspase-3 and γ-H2A.X were higher than the other groups, while Bcl-2 and survivin levels were greatly decreased [**Fig. 6(B)**]. It was noted that about 33% of the cells underwent DNA fragmentation (sub-G_1/G_0 peak), and the multiploidization effect was totally suppressed in this group [**Fig. 7(A,B)**]. These results demonstrate that combination of CuB and VPA could aggravate DNA damage, and induce cell apoptosis.

Discussion

In this study, we show that single-agent CuB inhibited the proliferation of melanoma cells in a dose-dependent manner. However, our data also clearly demonstrate that a compensatory response, as indicated by increased phospho-eIF2α and Bcl-2 levels, was induced by CuB treatment. Moreover, the induction of autophagy seemed to confer resistance of cells to CuB treatment, since the addition of the well-known autophagy inhibitor CQ to CuB-treated cells resulted in more cytotoxicity and apoptosis. Our observations in CuB-treated melanoma cells are supported by a large number of previous works that the induction of autophagy may protect cancer cells from drug-induced apoptosis [31]. It is therefore important to develop new strategy to overcome this pro-survival response induced by many antitumor agents.

The administration of CuB may greatly sensitize a variety of cancers to other anti-cancer agents. For example, the combination of CuB with cisplatin induces more apoptosis in pancreatic and laryngeal squamous cell carcinoma cells by downregulating Bcl-2 expression and STAT3 phosphorylation [6,32]. In this study, we evaluated whether HDAC inhibitor VPA and CuB had synergistic effects on melanoma cells. HDAC inhibitors such as suberoylanilide hydroxamic acid and VPA, which regulate a large number of genes functioning in cellular metabolism and cell survival, represent a new group of antitumor drugs [33]. The antitumor effect of VPA by induction of cell apoptosis has been reported in various cancers, such as small cell lung cancer, chronic lymphocytic leukemia, and hepatoma [17,34,35]. VPA treatment also sensitizes tumor cells to some first-line antitumor drugs, such as doxorubicin, cisplatin, and etoposide [16,17]. Consistent with these reports, our results demonstrated that a combination of CuB with VPA overcame the resistant compensatory response and multiploidization effect induced by CuB. By combination of CuB and VPA, the apoptotic pathway was activated in the melanoma cells, and a significant fraction of cells underwent apoptosis as evidenced by caspase-3 activation and induction of sub-G_0/G_1 peaks.

We observed that autophagy was also induced by combinatorial CuB and VPA. It is not clear whether the induced autophagy pathway contributes to the apoptosis in the CuB plus VPA-treated cells. Autophagy is regulated by several pathways, including AMP-activated protein kinase/mammalian target of rapamycin (mTOR), phosphatidylinositol 3-kinase/RAC-alpha serine/threonine-protein kinase (Akt)/mTOR and mitogen-activated protein kinase/extracellular signal-regulated kinase/mTOR [31]. Activation of JNKs facilitates the dissociation of Beclin-1 from Bcl-2/Beclin-1 complex, leading to the initiation of autophagy [36].

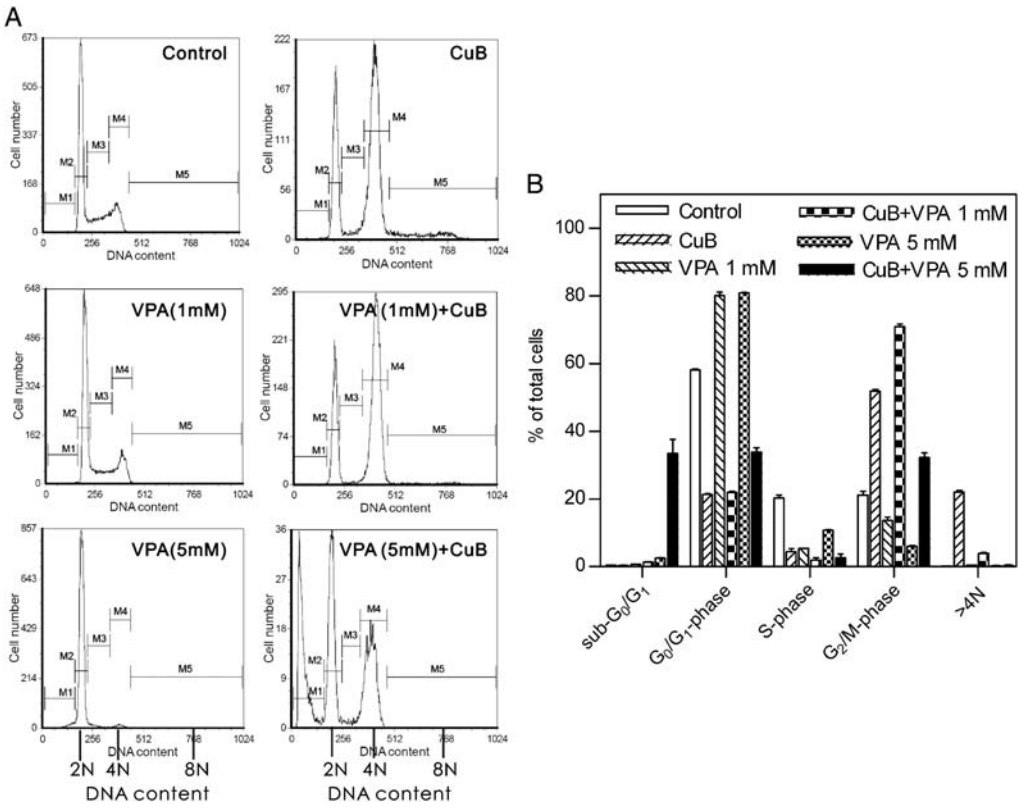

Figure 7 Cell cycle distribution and DNA fragmentation of B16F10 cells treated with CuB (1 μM) and/or VPA for 48 h After treatment, cells were stained with PI, and DNA contents were evaluated by flow cytometry. Both flow cytometric data (A) and histogram (B) are presented. Data in the histograms are means ± SD from one of the three independent experiments. The 2N, 4N, and 8N indicate cells with diploid, tetraploid (or binuclear), and eight-ploid (or four nuclear) DNA contents, respectively. M1, sub-G_1/G_0; M2, G_1/G_0; M3, S; M4, G2/M; M5: eight-ploid (>4N).

However, persistent JNK activation may also result in cell apoptosis. We presumed that it was the prolonged activation of JNKs that led to the increase of cell death under the combinatorial action of CuB and VPA. Other investigators have also demonstrated that the persistent activation of JNKs contributes to the cell death induced by cisplatin, 2-methoxyestradiol, and 12-otetradecanoyl-phorbol-13-acetate [37–40]. Consistent with these studies, we observed that sustained activation of JNKs by CuB plus VPA was associated with more DNA fragmentation and cell apoptosis, whereas transient activation of JNKs by CuB, was related to less cell death.

On the other hand, the activation of JNKs by mitogen-activated protein kinase kinase (MAPKK) MEKK1 contributes to the activation of STAT3 in different types of cells treated with growth factors, such as epidermal growth factor and platelet-derived growth factor [41]. STAT3 is a latent transcription factor that is well known to be activated by IL-6. The activation of STAT3 usually promotes cell proliferation and prevents cells from apoptosis. For example, most cancer cells, instead of normal ones, are characterized by constitutive activation of STAT3 [42]. In this study, CuB treatment also induced STAT3 activation in the melanoma cells (**Supplementary Fig. S2**), which was likely to protect cells from apoptosis. However, STAT3 activation may also block cell cycle progression by increasing the expression of *p21*$^{Waf1/Cip1}$ gene [43]. The activation of STAT3 and up-regulation of $p21^{Waf1/Cip1}$ expression by cucurbitacin glucosides are associated with cell cycle arrest and cell death [44]. Other investigators have indicated that VPA, as well as other short-chain fatty acid HDAC inhibitors, induces G_0/G_1 arrest by increasing the expression of $p21^{Waf1/Cip1}$ in a variety of cancers cells [45–47]. Although the $p21^{Waf1/Cip1}$ expression was not determined in this study, the G_0/G_1 arrest in the VPA-treated melanoma cells suggests that VPA treatment might increase the expression of $p21^{Waf1/Cip1}$ in this cell line. In addition, the effect of VPA on cell cycle arrest, which is likely due to an increase of $p21^{Waf1/Cip1}$ expression, might also account for the inhibitory effect of combinatorial CuB and VPA on multiploidization. In other words, VPA treatment arrested the bi-nuclear or tetraploid cells at G_0/G_1 phase, although we were unable to distinguish them from the G_2/M phase cells by flow cytometry. Furthermore, the addition of VPA enhanced the activation of STAT3 in CuB-treated melanoma cells (**Supplementary Fig. S2**). Previous studies

have revealed that sustained activation of both STAT3 and JNKs may result in cell apoptosis [48]. In addition, the enhancement of STAT3 activation and the downregulation of survivin expression were simultaneously observed in CuB and VPA-treated cells in this study. Taken together, we speculate that the sustained JNK activation may act in synergy with the enhanced STAT3 activation, and eventually lead to enhanced apoptotic cell death in the CuB plus VPA groups. However, it remains to be determined whether the enhanced STAT3 activation in the CuB plus VPA group is caused by sustained activation of JNKs.

In summary, VPA, an HDAC inhibitor, could act with CuB synergistically to inhibit the growth of melanoma cells and enhance the activation of apoptotic pathway. The combinatorial effects of CuB plus VPA were likely due to the sustained activation of both JNKs and STAT3, although the role of STAT3 activation in induction of cell apoptosis still needs to be clarified. Our results suggest that combination of CuB and HDAC inhibitors may provide a novel regimen against melanomas.

Supplementary Data

Supplementary data are available at *ABBS* online.

Funding

This work was supported by the Fundamental Research Funds for the Central Universities (21609403), the grants from the Major State Basic Research Development Program of China (973 Program) (2010CB833603), and the Natural Science Fund of Guangdong Province (8451063201000340).

References

1 Trinh VA. Current management of metastatic melanoma. Am J Health Syst Pharm 2008, 65: S3–S8.
2 Blaskovich MA, Sun J, Cantor A, Turkson J, Jove R and Sebti SM. 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 2003, 63: 1270–1279.
3 Wakimoto N, Yin D, O'Kelly J, Haritunians T, Karlan B, Said J and Xing H, *et al*. Cucurbitacin B has a potent antiproliferative effect on breast cancer cells *in vitro* and *in vivo*. Cancer Sci 2008, 99: 1793–1797.
4 Yin D, Wakimoto N, Xing H, Lu D, Huynh T, Wang X and Black KL, *et al*. Cucurbitacin B markedly inhibits growth and rapidly affects the cytoskeleton in glioblastoma multiforme. Int J Cancer 2008, 123: 1364–1375.
5 Haritunians T, Gueller S, Zhang L, Badr R, Yin D, Xing H and Fung MC, *et al*. Cucurbitacin B induces differentiation, cell cycle arrest, and actin cytoskeletal alterations in myeloid leukemia cells. Leuk Res 2008, 32: 1366–1373.
6 Zhang M, Sun C, Shan X, Yang X, Li-Ling J and Deng Y. Inhibition of pancreatic cancer cell growth by cucurbitacin B through modulation of signal transducer and activator of transcription 3 signaling. Pancreas 2010, 39: 923–929.
7 Liu T, Zhang M, Zhang H, Sun C and Deng Y. Inhibitory effects of cucurbitacin B on laryngeal squamous cell carcinoma. Eur Arch Otorhinolaryngol 2008, 265: 1225–1232.
8 Yasuda S, Yogosawa S, Izutani Y, Nakamura Y, Watanabe H and Sakai T. Cucurbitacin B induces G2 arrest and apoptosis via a reactive oxygen species-dependent mechanism in human colon adenocarcinoma SW480 cells. Mol Nutr Food Res 2010, 54: 559–565.
9 Chan KT, Meng FY, Li Q, Ho CY, Lam TS, To Y and Lee WH, *et al*. 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 2010, 294: 118–124.
10 Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R and Ciliberto G, *et al*. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 1999, 10: 105–115.
11 Garcia R and Jove R. Activation of STAT transcription factors in oncogenic tyrosine kinase signaling. J Biomed Sci 1998, 5: 79–85.
12 Aggarwal BB, Sethi G, Ahn KS, Sandur SK, Pandey MK, Kunnumakkara AB and Sung B, *et al*. Targeting signal-transducer-and-activator-of-transcription-3 for prevention and therapy of cancer: modern target but ancient solution. Ann N Y Acad Sci 2006, 1091: 151–169.
13 Richon VM, Sandhoff TW, Rifkind RA and Marks PA. Histone deacetylase inhibitor selectively induces $p21^{WAF1}$ expression and gene-associated histone acetylation. Proc Natl Acad Sci USA 2000, 97: 10014–10019.
14 Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW and Moon EJ, *et al*. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. Nat Med 2001, 7: 437–443.
15 Di GE, Bruzzese F, Pepe S, Leone A, Delrio P, Subbarayan PR and Avallone A, *et al*. Modulation of thymidilate synthase and p53 expression by HDAC inhibitor vorinostat resulted in synergistic antitumor effect in combination with 5FU or raltitrexed. Cancer Biol Ther 2009, 8: 782–791.
16 Wittenburg LA, Bisson L, Rose BJ, Korch C and Thamm DH. The histone deacetylase inhibitor valproic acid sensitizes human and canine osteosarcoma to doxorubicin. Cancer Chemother Pharmacol 2011, 67: 83–92.
17 Hubaux R, Vandermeers F, Crisanti MC, Kapoor V, Burny A, Mascaux C and Albelda SM, *et al*. Preclinical evidence for a beneficial impact of valproate on the response of small cell lung cancer to first-line chemotherapy. Eur J Cancer 2010, 46: 1724–1734.
18 Kristensen LS, Nielsen HM and Hansen LL. Epigenetics and cancer treatment. Eur J Pharmacol 2009, 625: 131–142.
19 Bacon CL, Gallagher HC, Haughey JC and Regan CM. Antiproliferative action of valproate is associated with aberrant expression and nuclear translocation of cyclin D3 during the C6 glioma G1 phase. J Neurochem 2002, 83: 12–19.
20 Batty N, Malouf GG and Issa JP. Histone deacetylase inhibitors as antineoplastic agents. Cancer Lett 2009, 280: 192–200.
21 Chan KT, Li K, Liu SL, Chu KH, Toh M and Xie WD. Cucurbitacin B inhibits STAT3 and the Raf/MEK/ERK pathway in leukemia cell line K562. Cancer Lett 2010, 289: 46–52.
22 Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA and Klein PS. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 2001, 276: 36734–36741.
23 Kramer OH, Zhu P, Ostendorff HP, Golebiewski M, Tiefenbach J, Peters MA and Brill B, *et al*. The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. EMBO J 2003, 22: 3411–3420.
24 Gottlicher M. Valproic acid: an old drug newly discovered as inhibitor of histone deacetylases. Ann Hematol 2004, 83 Suppl 1: S91–S92.

25 Wek RC, Jiang HY and Anthony TG. Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans 2006, 34: 7–11.
26 Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H and Ogawa S, *et al*. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ 2007, 14: 230–239.
27 Karna P, Zughaier S, Pannu V, Simmons R, Narayan S and Aneja R. Induction of reactive oxygen species-mediated autophagy by a novel microtubule-modulating agent. J Biol Chem 2010, 285: 18737–18748.
28 Eum KH and Lee M. Crosstalk between autophagy and apoptosis in the regulation of paclitaxel-induced cell death in v-Ha-ras-transformed fibroblasts. Mol Cell Biochem 2011, 348: 61–68.
29 Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M and Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 2000, 10: 886–895.
30 Rogakou EP, Pilch DR, Orr AH, Ivanova VS and Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 1998, 273: 5858–5868.
31 Chen S, Rehman SK, Zhang W, Wen A, Yao L and Zhang J. Autophagy is a therapeutic target in anticancer drug resistance. Biochim Biophys Acta 2010, 1806: 220–229.
32 Liu T, Peng H, Zhang M, Deng Y and Wu Z. Cucurbitacin B, a small molecule inhibitor of the Stat3 signaling pathway, enhances the chemosensitivity of laryngeal squamous cell carcinoma cells to cisplatin. Eur J Pharmacol 2010, 641: 15–22.
33 Marks PA. The clinical development of histone deacetylase inhibitors as targeted anticancer drugs. Expert Opin Investig Drugs 2010, 19: 1049–1066.
34 Bokelmann I and Mahlknecht U. Valproic acid sensitizes chronic lymphocytic leukemia cells to apoptosis and restores the balance between pro- and antiapoptotic proteins. Mol Med 2008, 14: 20–27.
35 Armeanu S, Pathil A, Venturelli S, Mascagni P, Weiss TS, Gottlicher M and Gregor M, *et al*. Apoptosis on hepatoma cells but not on primary hepatocytes by histone deacetylase inhibitors valproate and ITF2357. J Hepatol 2005, 42: 210–217.
36 Bogoyevitch MA, Ngoei KR, Zhao TT, Yeap YY and Ng DC. c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges. Biochim Biophys Acta 2010, 1804: 463–475.
37 Lorin S, Borges A, Ribeiro Dos SL, Souquere S, Pierron G, Ryan KM and Codogno P, *et al*. c-Jun NH2-terminal kinase activation is essential for DRAM-dependent induction of autophagy and apoptosis in 2-methoxyestradiol-treated Ewing sarcoma cells. Cancer Res 2009, 69: 6924–6931.
38 Mansouri A, Ridgway LD, Korapati AL, Zhang Q, Tian L, Wang Y and Siddik ZH, *et al*. Sustained activation of JNK/p38 MAPK pathways in response to cisplatin leads to Fas ligand induction and cell death in ovarian carcinoma cells. J Biol Chem 2003, 278: 19245–19256.
39 Chen Y, Wu Q, Song SY and Su WJ. Activation of JNK by TPA promotes apoptosis via PKC pathway in gastric cancer cells. World J Gastroenterol 2002, 8: 1014–1018.
40 Sanchez-Perez I, Murguia JR and Perona R. Cisplatin induces a persistent activation of JNK that is related to cell death. Oncogene 1998, 16: 533–540.
41 Lim CP and Cao X. Regulation of Stat3 activation by MEK kinase 1. J Biol Chem 2001, 276: 21004–21011.
42 Aggarwal BB, Kunnumakkara AB, Harikumar KB, Gupta SR, Tharakan ST, Koca C and Dey S, *et al*. Signal transducer and activator of transcription-3, inflammation, and cancer: how intimate is the relationship? Ann N Y Acad Sci 2009, 1171: 59–76.
43 Barre B, Avril S and Coqueret O. Opposite regulation of myc and $p21^{waf1}$ transcription by STAT3 proteins. J Biol Chem 2003, 278: 2990–2996.
44 Tannin-Spitz T, Grossman S, Dovrat S, Gottlieb HE and Bergman M. Growth inhibitory activity of cucurbitacin glucosides isolated from Citrullus colocynthis on human breast cancer cells. Biochem Pharmacol 2007, 73: 56–67.
45 Das CM, Aguilera D, Vasquez H, Prasad P, Zhang M, Wolff JE and Gopalakrishnan V. Valproic acid induces p21 and topoisomerase-II (alpha/beta) expression and synergistically enhances etoposide cytotoxicity in human glioblastoma cell lines. J Neurooncol 2007, 85: 159–170.
46 Rocchi P, Tonelli R, Camerin C, Purgato S, Fronza R, Bianucci F and Guerra F, *et al*. $p21^{Waf1/Cip1}$ is a common target induced by short-chain fatty acid HDAC inhibitors (valproic acid, tributyrin and sodium butyrate) in neuroblastoma cells. Oncol Rep 2005, 13: 1139–1144.
47 Jung GA, Yoon JY, Moon BS, Yang DH, Kim HY, Lee SH and Bryja V, *et al*. Valproic acid induces differentiation and inhibition of proliferation in neural progenitor cells via the beta-catenin-Ras-ERK-$p21^{Cip/WAF1}$ pathway. BMC Cell Biol 2008, 9: 66.
48 Ahmed-Choudhury J, Williams KT, Young LS, Adams DH and Afford SC. CD40 mediated human cholangiocyte apoptosis requires JAK2 dependent activation of STAT3 in addition to activation of JNK1/2 and ERK1/2. Cell Signal 2006, 18: 456–468.