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Full Length Research Paper

Vitexicarpin induces apoptosis-independent mitotic arrest in U87 glioblastoma cells

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Glioblastoma multiforme is the most common and lethal primary brain tumor that responds poorly to currently available chemotherapy. Vitexicarpin, a flavonoid compound has been reported to exhibit anti-proliferative activities against various cancer cell lines. However, the anticancer effect of vitexicarpin on glioblastoma remains unexplored. In the present study, we found that vitexicarpin inhibited the growth of U87 glioblastoma cells in a dose-dependent manner with IC_{50} ~22 µM. Vitexicarpin-induced growth inhibition was found to be associated with induction of apoptosis and mitotic arrest. During vitexicarpin-induced apoptosis, up-regulation of Bax, down-regulation of Bcl-2 and cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP) were observed. We also found that vitexicarpin induced mitotic arrest by inhibiting tubulin polymerization. Furthermore, pretreatment of cells with z-VAD-fmk reversed the apoptotic effect of vitexicarpin but failed to attenuate mitotic arrest. Taken together, our data revealed that vitexicarpin inhibited the growth of U87 cells by induction of apoptosis and mitotic arrest. Thus, vitexicarpin may be a promising candidate for the treatment of glioblastoma.

Key words: Vitexicarpin, glioblastoma, mitotic arrest, apoptosis, caspase-3.

INTRODUCTION

Primary brain tumors are the neoplasms that originate from various intracranial tissues. More than 60% of brain tumors are gliomas (Yin et al., 2008). Glioblastoma multiforme is the most common and lethal primary brain tumor in adults and accounts for at least 80% of malignant gliomas and 20% of all brain neoplasms (Chen et al., 2010; Zhu et al., 2011; Amantini et al., 2007; Su et al., 2008). It is also called grade IV astrocytoma (Das et al., 2010). Over 12,000 patients die because of primary brain tumor in United States every year. Despite recent advances in surgical therapy, radiation-therapy and chemotherapy, it is still difficult to effectively treat glioblastoma and the median survival rate remains less than one year after diagnosis (Fujita et al., 2008; Jeon et al., 2008; Yin et al., 2008).

Flavonoids, a group of phenolic compounds, have multiple biological, pharmacological and medicinal properties, including anti-inflammatory, anti- viral, anti-allergic, anti-thrombotic and anti-cancer effects (de Sampaio e Spohr et al., 2010; Kim et al., 2008). Vitexicarpin is one of the major flavonoid compounds isolated from the dried fruits of *Vitex rotundifolia.* It has been extensively used in Chinese traditional medicines as an anti-inflammatory drug for thousands of years. In recent years, it has been reported to exhibit anti-carcinogenic activity in a few human cancer cell lines (Haidara et al., 2006; Kobayakawa et al., 2004; Shen et al., 2009). Further studies demonstrated that vitexicarpin induced cell death in K562 cells through mitotic catastrophe and induction of apoptosis via PI3K/AKT signalling pathway, through G2/M arrest and Bax/Bcl-2

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pathway in human pancreatic carcinoma PANC-1 cells, human breast cancer cells MN1 and MDD2 and through G2/M arrest in human epidermoid carcinoma cells KB and A431 (Ding et al., 2012; Haidara et al., 2006; Kobayakawa et al., 2004; Shen et al., 2009). However, no report is at present available on anti-glioma activity of vitexicarpin.

The purpose of present study was (i) to examine whether vitexicarpin inhibits growth of brain tumor cells using U87 glioblastoma cell line, (ii) to examine whether apoptosis and cell cycle arrest are involved in cell growth inhibition and (iii) to examine the relationship between apoptosis and cell cycle arrest and their molecular mechanism.

MATERIALS AND METHODS

Chemicals and reagents

Vitexicarpin/casticin (Figure 1A) was purchased from Tauto Biotech Co., Ltd. (Shanghai, China). RNase A, propidium iodide (PI) calcein acetoxymethylester (Calcein AM), dimethyl sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), Dulbecco's modified eagle's medium (DMEM) and fatal bovine serum (FBS), were purchased from Sigma (Beijing, China). Apoptosis assay kit, general caspase inhibitor (z-VAD-fmk), antibodies specific to Bax, Bcl-2, caspase-3, poly(ADP-ribose) polymerase (PARP) and tubulin were purchased from Beyotime Institute of Technology (Haimen, Jiangsu, China). Antibodies specific to β-actin and horseradish peroxidase-conjugated secondary antibodies (goat-anti-rabbit, goat-anti-mouse) were purchased from Santa Cruz Biotechnology, Inc. (Delaware Ave, USA).

Cell culture

U87 glioblastoma cells were obtained from American Type Culture Collection (ATCC, USA) and maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C. H1299 cells were obtained from Shanghai Cell Bank and maintained in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C. Cells were treated with various concentrations of vitexicarpin (purity 99.5%) dissolved in DMSO with a final DMSO concentration of 1% or with DMSO alone for 24 h. DMSO treated cells were used as control.

Determination of cell viability

The effect of vitexicarpin on cell viability was measured by MTT assay as described previously (Ding et al., 2012). Briefly, U87 cells were treated with various concentrations of vitexicarpin (1 to 80 µM) for 24 h. Following treatment, the MTT reagent was added (500 µg/ml) and cells were further incubated at 37°C for 4 h. Subsequently, 150 µL DMSO was added to dissolve farmazan crystals and absorbance was measured at 570 nm in a microplate reader (Thermo Scientific). Results were expressed as the percentage of MTT reduction, assuming that the absorbance of control cells was 100%.

Cell morphological examination by light microscopy

U87 cells were treated with vitexicarpin (10 and 20 µM) for 24 h. Morphological changes were observed by phase contrast microscopy (Olympus 1x71).

Live/dead cell assay

U87 glioblastoma cells were treated with 10 and 20 µM of vitexicarpin for 24 h. Live and dead cells were quantified using fluorescent probes calcein AM and PI as previously described (Khan et al., 2012). Calcein AM is cell membrane permeable. In viable cells, it is converted into calcein by esterases and emits strong green fluorescence. PI, a nuclei staining dye, is cell membrane impermeable. Cells with impaired plasma membrane integrity are stained red due to entry of PI and failure to retain calcein. Since both calcein and PI can be excited with 490 nm, simultaneous monitoring of viable and dead cells is possible with a fluorescence microscope. After treatment, floating and adherent cells were collected, rinsed with PBS and incubated with PBS solution containing 2 µM calcein AM and 4 µM PI in the dark for 20 min at room temperature. At the end, 100 cells were counted microscopically for the percentage of live and dead cells.

Flow cytometry analysis of apoptosis

U87 cells were treated with 10 and 20 µM vitexicarpin for 24 h. After treatment, both adherent and floating cells were collected, washed with PBS and resuspended in 200 µL of binding buffer containing 5 µL Annexin V and put in the dark for 10 min according to the kit instructions (Beyotime, Jiangsu, China). After incubation, cell were labeled with 10 µL PI and samples were immediately analyzed by flow cytometry (Beckman Coulter, Epics XL).

Flow cytometry analysis of cell cycle

U87 and H1299 cells were treated with 10 and 20 µM vitexicarpin in the presence or absence of z-VAD-fmk or PFT-α for 24 h. Following treatment, cells were harvested, washed with PBS and fixed with 70% ethanol on ice for 20 min. After washing twice with PBS, cells were stained with a solution containing 50 µg/ml PI and 100 µg/ml RNase A for 30 min in the dark, at room temperature. Cell cycle phase distribution and DNA contents were analyzed by flow cytometry (Beckman Coulter, Epics XL).

Protein extraction and Western blotting

After treatment, adherent and floating cells were collected and proteins were isolated as described by us previously (Khan et al., 2012). Briefly, 50 µg proteins were subjected to electrophoresis on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. After blocking with 5% (w/v) non-fat milk and washing with tris-buffered saline-Tween solution (TBST), membranes were incubated overnight at 4°C with PARP (1:1000), BCL-2 (1:1000), Bax (1:300), caspase-3 (1:500), tubulin (1:500) and β-actin (1:400), antibodies respectively. After washing, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary antibodies (1:1000) for 1 h at room temperature. After washing with TBST, signals were detected using ECL plus chemiluminescence kit (Millipore Corporation) on X-ray film.

Extraction of monomeric and polymeric tubulin

After treating the cells with 20 µM vitexicarpin, cells were harvested, washed with PBS and monomeric and polymeric tubulin were

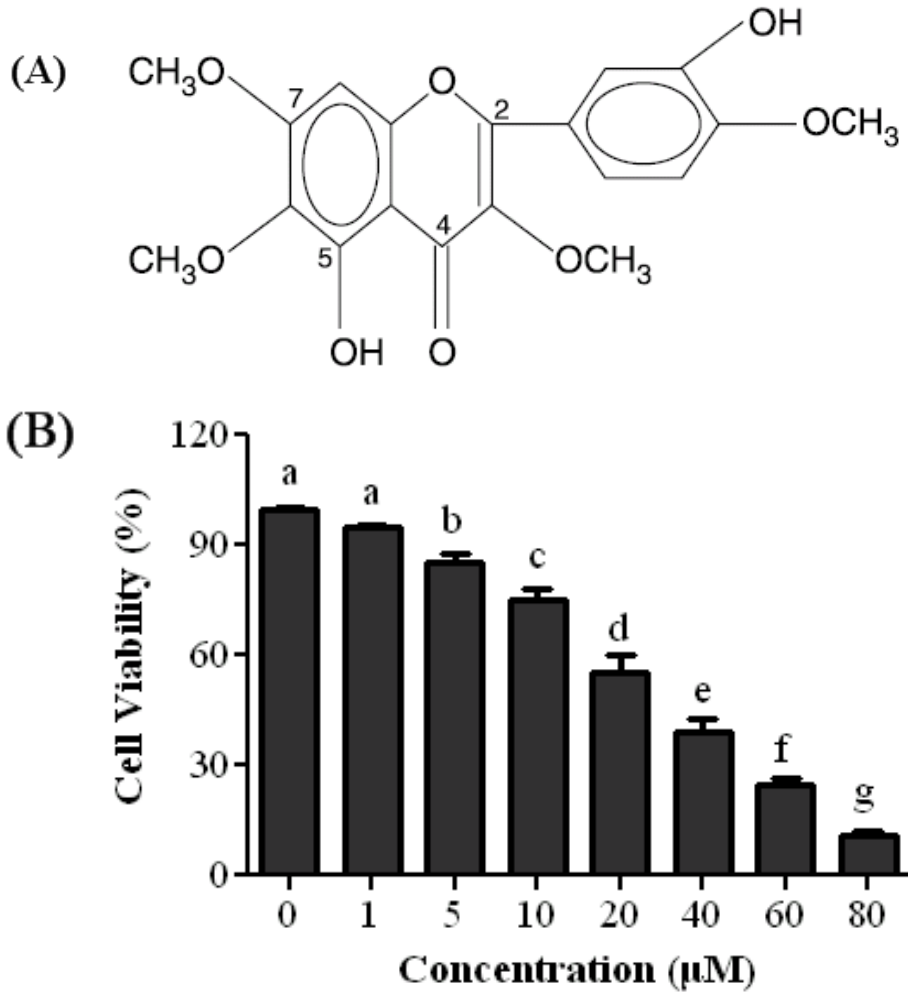

Figure 1. Chemical structure of vitexicarpin and its effect on cell viability. (A) Chemical structure of vitexicarpin. (B) U87 cells were treated with various concentrations of vitexicarpin for 24 h and cell viability was determined by MTT assay. Data are expressed as mean ± SEM (n=3). Columns not sharing the same superscript letter differ significantly ($P<0.05$)

extracted as described previously (Shen et al., 2009). Briefly, the cell pellet was resuspended in 0.4 ml monomeric extraction buffer [20 mM Tris–HCl, pH 6.8, 0.14 M NaCl, 1 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.5% Nonidet P-40 (NP-40), 0.5 mM phenylmethanesulfonyl fluoride (PMSF) and 4 µg/ml paclitaxel], centrifuged at 12,000×g for 10 min and supernatant containing monomeric tubulin was transferred to a new tube. The pellets containing polymeric tubulin were resuspended in WIP cell lysis reagent (BIOSS, Beijing Biosynthesis Biotechnology Co., LTD.) for 30 min and the supernatant (polymeric tubulin) was collected by centrifugation at 12,000 × g for 10 min. The monomeric and polymeric tubulins were subjected to Western blot analysis.

Statistical analysis

The results are expressed as Mean ± SEM of three independent experiments and statistically compared with control group or within the groups using one way ANOVA followed by Tukey's multiple comparison test.

RESULTS

Cytotoxic effect of vitexicarpin on U87 glioblastoma cells

Cytotoxic effect of vitexicarpin on U87 glioblastoma cells was determined by MTT assay, Live/dead cell assay and observing morphological changes of cells. Treatment with vitexicarpin for 24 h, inhibited growth of U87 cells in a dose-dependent manner as shown in Figure 1B. The IC_{50} of vitexicarpin was 22 µM after 24 h treatment. Morphological changes were observed by phase contrast microscopy. After treatment with vitexicarpin, a decrease in total number of cells and an increase in floating cells were observed in culture medium. Cell treated with vitexicarpin exhibited characteristic morphological changes of cell death including rounding and shrinkage

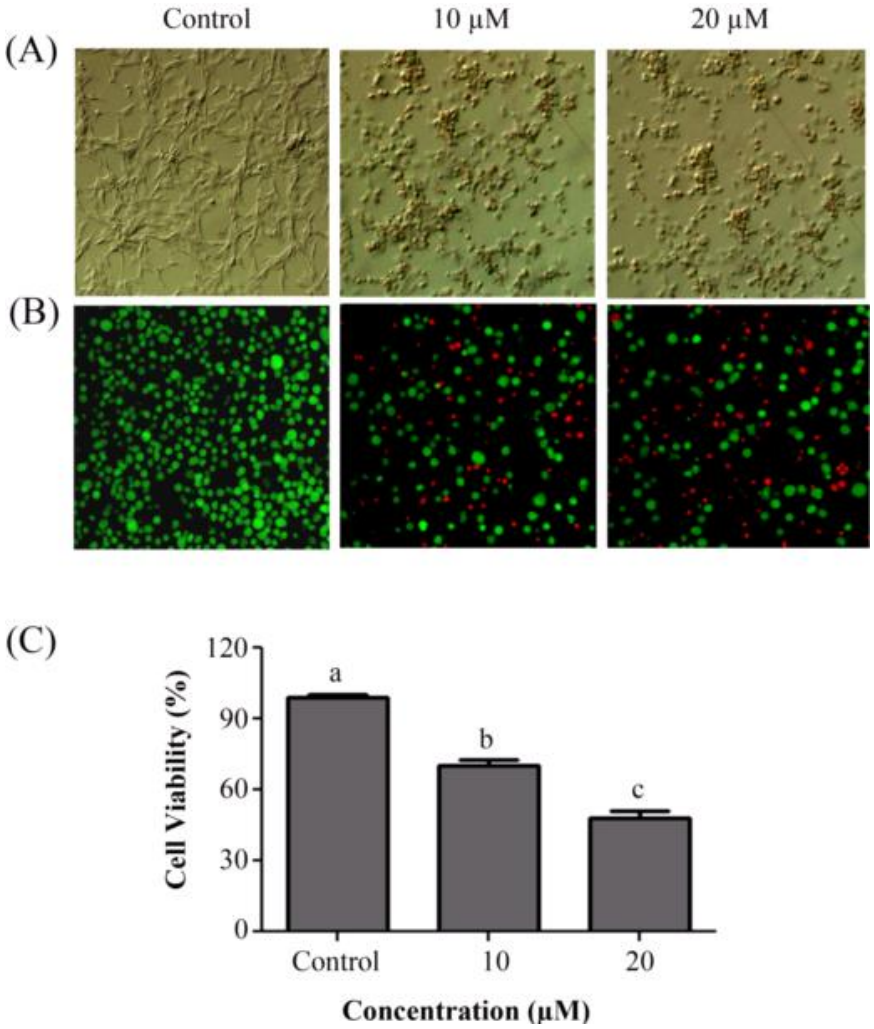

Figure 2. Microscopic study of U87 cells (A) U87 cells were treated with 10 and 20 μM vitexicarpin for 24 h and morphological changes were observed with phase contrast microscopy (Scale bar = 100 μM). (B) U87 cells were treated with 10 and 20 μM vitexicarpin for 24 h and live and dead cells were observed using fluorescent probes calcein AM and PI and fluorescence microscopy. Viable cells stained green, while dead cells stained red. (C) Statistical analysis of cell viability by Live/dead assay. Data are expressed as mean ± SEM (n=3). Columns not sharing the same superscript letter differ significantly (P<0.05).

as shown in Figure 2A. Furthermore, the live and dead cells were quantified by Live/dead assay. As shown in Figure 2B and C, vitexicarpin treatment reduced the viability of cells in a dose-dependent manner. The viability of cells treated with 10 and 20 μM vitexicarpin for 24 h was significantly lower (70 ± 2.64 and 48.33 ± 2.84, respectively vs. 99 ± 0.99 in control group, P<0.05).

Vitexicarpin induces apoptosis in U87 glioblastoma cells

Apoptotic cell death was assessed by Annexin V-FITC and PI staining. Treatment of cells with 10 and 20 μM vitexicarpin significantly increased apoptosis rate (24.7±2.83 and 37.4±1.83% vs. 1.18±0.43% in control group, P<0.05) (Figure 3). Pretreatment with general caspase inhibitor (z-VAD-fmk) attenuated the apoptotic effect of vitexicarpin significantly (Figure 3D). The data clearly demonstrated that vitexicarpin induced caspase-dependent apoptosis in U87 glioblastoma cells.

Vitexicarpin induces mitotic arrest in U87 glioblastoma cells

Cell cycle arrest and apoptosis are the main causes of cell growth inhibition (Chan et al., 2010). To determine whether the cell growth inhibition involved growth arrest at a specific phase of cell cycle, flow cytometric analysis

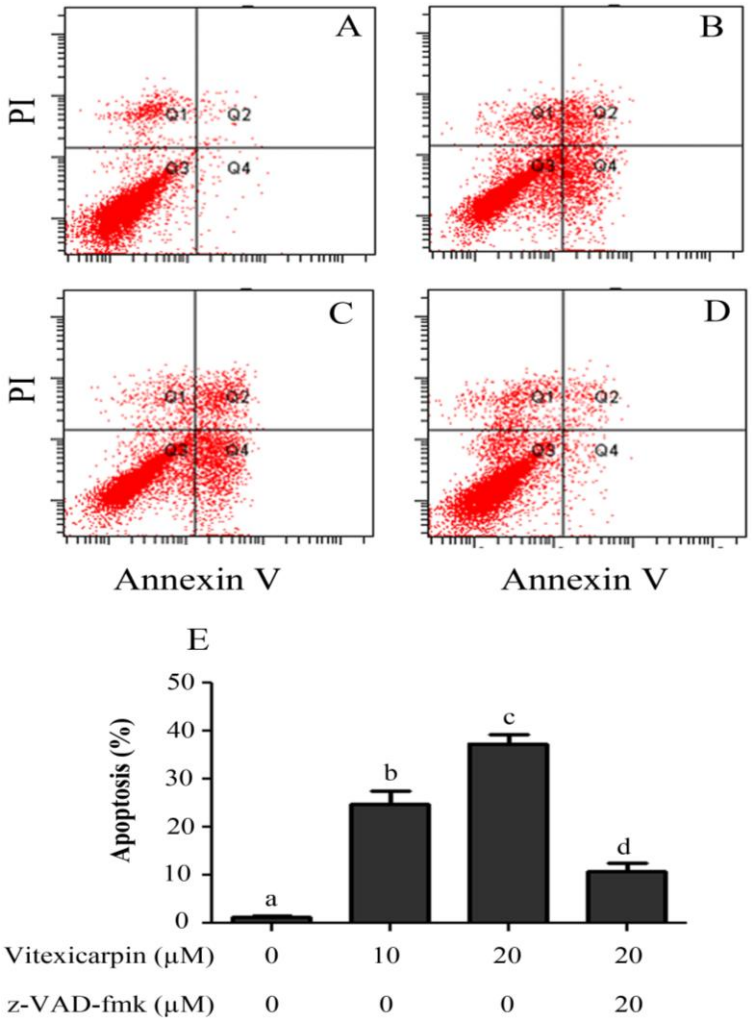

Figure 3. Flow cytometry analysis of apoptosis in U87 glioblastoma cells treated with 10 and 20 µM vitexicarpin in the presence or absence of 20 µM z-VAD-fmk for 24 h. (A) Control; (B and C) U87 cells were treated with 10 and 20 µM vitexicarpin for 24 h. (D) U87 cells were treated with 20 µM vitexicarpin in the presence of 20 µM z-VAD-fmk for 24. Cells were collected by centrifugation, washed with PBS and stained with Annexin V/PI according to kit instructions. When the cells are double stained with annexin V-FITC and PI, four different populations of cells can be observed. The cells that do not stain with either annexin V or PI are alive and reside in region Q3; the cells that stain with only annexin V are in the stage of early apoptosis and reside in region Q4; the cells that stain with both reagents are nonviable apoptotic/necrotic cells and scatter in region Q2, while region Q1 represents the nuclear fragments/necrotic cells stained with only PI. (E) Data are expressed as mean ± SEM (n=3). Columns not sharing the same superscript letter differ significantly ($P<0.05$).

of cell cycle phase distribution was performed. The data showed that vitexicarpin arrested the cell cycle at G2/M phase in a dose-dependent manner (Figure 4). Treatment of cells with vitexicarpin at 10 and 20 µM showed a significant increase in G2/M phase from 10.66 ± 1.45 to 41.66 ± 1.76% and 58.66 ± 2.72%, respectively with a concomitant decrease in G0/G1 phase without any significant change in S phase (Figure 4A and D).

Vitexicarpin induces apoptosis-independent cell cycle arrest in U87 glioblastoma cells

To further establish a link between apoptosis and cell cycle arrest, we performed apoptosis and cell cycle analysis using a general caspase inhibitor, (z-VAD-fmk). As shown in Figure 3, caspase inhibitor significantly inhibited apoptosis rate but did not prevent mitotic arrest

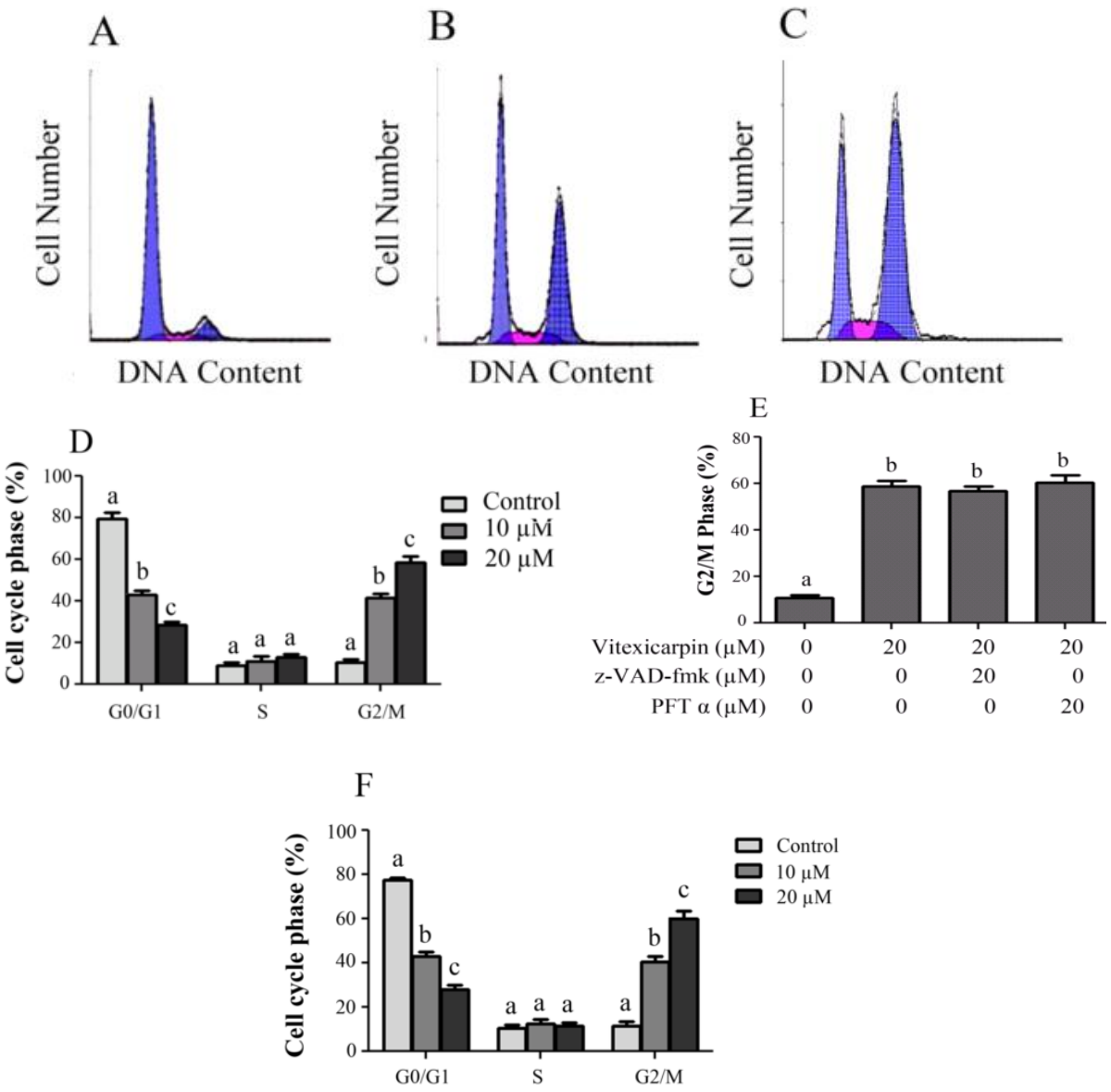

Figure 4. Flow cytometry analysis of cell cycle in U87 cells treated with 10 and 20 μM vitexicarpin in the presence or absence of 20 μM z-VAD-fmk or PFT-α for 24 h. (A) control; (B and C) U87 cells were treated with 10 and 20 μM vitexicarpin for 24 h. (D) Data are expressed as mean ± SEM (n=3). Columns not sharing the same superscript letter differ significantly (P<0.05). (E) U87 cells were treated with 20 μM vitexicarpin in the presence or absence of 20 μM z-VAD-fmk or 20 μM PFT-α for 24 h. Data are expressed as mean ± SEM (n=3). Columns not sharing the same superscript letter differ significantly (P<0.05). (F) H1299 cells were treated with 10 and 20 μM vitexicarpin for 24 h and cell cycle profile was analyzed by flow cytometry. Data are expressed as Mean ± SEM (n=3). Columns not sharing the same superscript letter differ significantly (P<0.05).

(Figure 4). The data suggest that cell cycle arrest induced by vitexicarpin in U87 glioblastoma cells is an apoptosis-independent and early event in cells death mediated by vitexicarpin.

Vitexicarpin induces mitotic arrest by inhibiting tubulin polymerization

Many anticancer agents arrest the cell cycle at G2/M phase either by damaging DNA or by disrupting mitotic spindles (Castedo et al., 2004; Ha et al., 2007; Orren et al., 1997). To investigate whether the inhibition of tubulin polymerization is involved in vitexicarpin-induced mitotic arrest, we extracted polymeric and monomeric tubulin from vitexicarpin-treated and untreated U87 glioblastoma cells. The expressions of polymeric and monomeric tubulin were observed by Western blot analysis. The results showed that vitexicarpin inhibited polymeric tubulin in U87 cells (Figure 5). Next, we wished to know

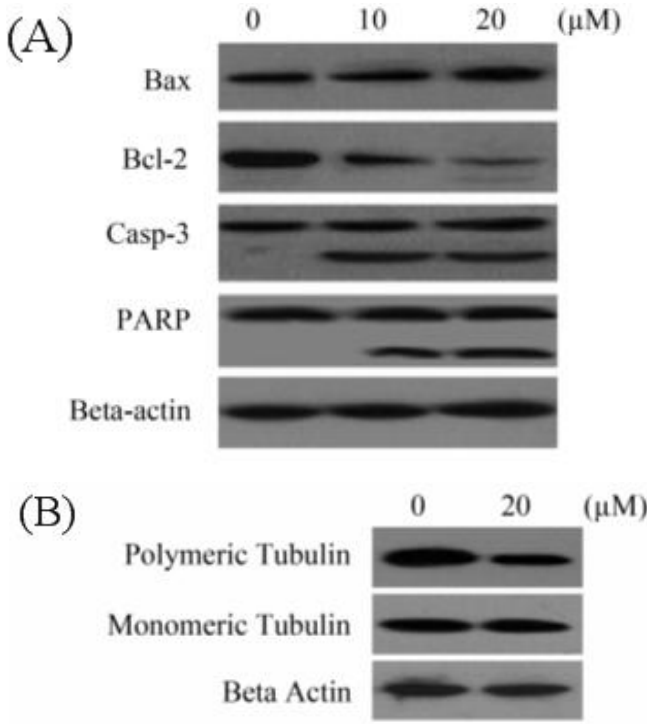

Figure 5. Effect of vitexicarpin on cell cycle and apoptosis regulators in U87 cells. (A) U87 cells were treated with 10 and 20 μM vitexicarpin for 24 h. The expression of Bax, Bcl-2, caspase-3 and PARP was determined by Western blot analysis. (B). U87 cells were treated with 20 μM vitexicarpin for 24 h. The expression of monomeric and polymeric tubulin was determined by Western blot analysis

whether p53 is also involved in G2/M arrest mediated by vitexicarpin. For this, we treated U87 cells with vitexicarpin in the presence of a specific p53 inhibitor (PFT-α) and cell cycle profile was analyzed by flow cytometry. The data showed that PFT-α did not attenuate mitotic arrest in U87 cells. The result indicated that vitexicarpin induced p53-independent mitotic arrest in U87 cells.

To further confirm p53-independent mitotic arrest, we treated H1299 cells ($p53^{-/-}$) with 10 and 20 μM vitexicarpin for 24 h and cell cycle profile was analyzed by flow cytometry. The data showed that vitexicarpin arrested the cell cycle of H1299 cells at G2/M phase in a similar fashion (Figure 4F). These results demonstrated clearly that vitexicarpin induces p53-independent mitotic arrest in U87 glioblastoma cells. In short, the data suggest that vitexicarpin arrests the cell cycle at Mitotic phase but not at G2 phase.

Vitexicarpin induces apoptosis in U87 cells through Bax/Bcl-2 pathway

It has been reported that mitotic spindle damage induces pro-apoptotic stimuli through mitochondrial pathway (Zuco and Zunino, 2008). To investigate the possible mechanism of apoptosis induced by vitexicarpin in U87 glioblastoma cells, we observed the expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 using Western blots. Our results demonstrated that vitexicarpin markedly increased the expression of Bax and decreased the expression of Bcl-2 in a dose-dependent manner (Figure 5). Mitochondrial-dependent apoptosis is initiated by recruitment and activation of caspases. Thus, we analyzed whether caspase-3 was activated during vitexicarpin-induced apoptosis of U87 glioblastoma cells. As shown in Figure 5, vitexicarpin stimulated the cleavage of caspase-3 in a dose-dependent manner as demonstrated by the appearance of 17 kDa caspase-3 active fragment. In addition, exposure of U87 glioblastoma cells to vitexicarpin strongly stimulated the cleavage of PARP, a substrate protein of caspase (Figure 5).

DISCUSSION

In the present study, we found that vitexicarpin effectively inhibited the growth and induced apoptosis and cell cycle arrest at Mitotic phase in U87 glioblastoma cells. Vitexicarpin has been reported to induce apoptosis and cell cycle arrest in human breast cancer cells, myeloid leukemia cells and epidermoid carcinoma cells (Haidara et al., 2006; Kobayakawa et al., 2004; Shen et al., 2009). However, the link between apoptosis and cell cycle arrest has not been fully established. Furthermore, the effect of vitexicarpin on glioma has not been studied. The present study was therefore conducted to investigate whether vitexicarpin induces a similar growth inhibitory effect on glioma cells; and whether this growth inhibitory effect is resulted from cell cycle arrest at a particular checkpoint of cell cycle or apoptosis.

In agreement with previous reports (Haidara et al., 2006; Kobayakawa et al., 2004; Shen et al., 2009), vitexicarpin inhibited the growth of U87 glioblastoma cells in a dose-dependent manner. Next, we wished to know whether this effect is from induction of apoptosis or cell cycle arrest. For this purpose, we performed flow cytometric analysis of apoptosis and cell cycle. The data showed that vitexicarpin induced apoptosis and arrested the cell cycle at G2/M phase in U87 glioblastoma cells. These results are consistent with previous studies that vitexicarpin induced apoptosis and G2/M phase arrest in other cancer cell lines (Haidara et al., 2006; Kobayakawa et al., 2004; Shen et al., 2009).

Apoptosis, a form of programmed cell death, is highly organized and evolutionary conserved process characterized by membrane blebbing, DNA frag-mentation, loss of plasma membrane integrity, activation of proteases and endonucleases, enzymatic cleavage of DNA into oligonucleosomal fragments and cleavage of PARP, a substrate protein of caspases (Gong et al., 2004; Lee

et al., 2000; Lin, 2001). In the present study, vitexicarpin-treated cells presented some common features of apoptosis such as translocation of phosphatidylserine from internal cell surface to external cell surface and cleavage of caspase-3. Our results are in agreement with previous studies (Shen et al., 2009). The apoptotic cell death can be divided into three main categories; (i) mitochondrial caspase-dependent apoptosis or intrinsic apoptosis, (ii) extrinsic apoptosis and (iii) caspase-independent apoptosis (Zhou et al., 2010). To gain further insight into molecular mechanism of vitexicarpin-induced apoptosis, we performed flow cytometric analysis of apoptosis using Annexin V/PI staining in the presence of pancaspase inhibitor z-VAD-fmk. Treatment of cells with z-VAD-fmk inhibited the apoptotic effect of vitexicarpin significantly suggesting that vitexicarpin induces caspase-dependent apoptosis in U87 glioblastoma cells.

Bcl-2 family proteins are involved in mitochondrial caspase-dependent apoptosis and play a central role in cell survival or cell death. The members of Bcl-2 family can function either as pro-apoptotic or as anti-apoptotic molecules. Under normal conditions, Bax, a pro-apoptotic protein is present in the cytosol and is negatively regulated by anti-apoptotic protein Bcl-2. Thus, Bcl-2/Bax is considered as molecular rheostat determining cell survival/death. In the presence of apoptotic stimulus, Bcl-2/Bax ratio decreases and cell undergoes apoptosis (Ji et al., 2011; Nathwani et al., 2010; Zhou et al., 2010; Antonsson et al., 2001; Ji et al., 2008). In the present study, remarkable increase in Bax expression and a dramatic decrease in Bcl-2 expression were observed in vitexicarpin-treated U87 glioblastoma cells in a dose-dependent manner. These results are consistent with previous study in which vitexicarpin induced apoptosis in K562 cell via mitochondrial caspase-dependent apoptosis (Haidara et al., 2006).

Caspases are also recognized as key mediators of apoptosis. Among the identified caspases, caspase-3 is the best characterized effector caspase, which is in the downstream of Bcl-2 and Bax. Cleavage of caspase-3 is a hallmark in the process of apoptosis. Once activated, caspase-3 leads to the final stage of cellular death by proteolytic cleavage of PARP and DNA on the one hand and activation of pro-apoptotic factors on the other hand (Gong et al., 2004; Kim et al., 2007; Yang et al., 2009). Thus we examined the expression of caspase-3 and PARP in U87 glioblastoma cells after exposure to vitexicarpin, by Western blot analysis. We found that vitexicarpin treatment stimulated the cleavage of caspase-3 and PARP in a dose-dependent manner. The data further confirmed that vitexicarpin promoted apoptosis through caspase-3 activation via mitochondrial pathway.

Cell cycle control is one of the major regulatory mechanisms of cell growth. Many anticancer compounds have been shown to arrest the cell cycle at G0/G1, S or G2/M checkpoints and thereby induce apoptosis (Gamet-Payrastre et al., 2000; Lu et al., 2007; Murray, 2004). In agreement with previous studies (Haidara et al., 2006; Kobayakawa et al., 2004; Shen et al., 2009), vitexicarpin arrested the cell cycle at G2/M phase in U87 cells in a dose-dependent manner. Many anticancer agents arrest the cell cycle at G2/M checkpoint either by damaging DNA or by disrupting mitotic spindle (Castido et al., 2004; Ha et al., 2007; Orren et al., 1997). P53, a tumor suppressor protein has been shown to arrest the cell cycle at G2/M checkpoint when DNA is damaged (Zuco and Zunino, 2008).

Next, we asked whether vitexicarpin arrested the cell cycle at G2/M phase by damaging DNA or by disrupting mitotic spindles. To answer the question, we observed the expression of monomeric and polymeric tubulin in cells of control and treatment groups using Western blot analysis. The data showed that vitexicarpin markedly inhibited tubulin polymerization which indicated that Mitotic arrest in U87 cells is associated with inhibition of tubulin polymerization. This result concurs with those of Kobayakaw et al. (2004) Shen et al. (2009) and Haidara et al. (2006) who demonstrated a similar effect of vitexicarpin on human epidermoid carcinoma KB cell line, human myeloid leukemia K562 cell line and breast cancer MN1 and MDD2 cell lines. We also asked whether cell cycle arrest is apoptosis-dependent. To address this question, we performed cell cycle analysis in the presence of caspase inhibitor, z-VAD-fmk. The data demonstrated that treatment of cells with z-VAD-fmk did not prevent mitotic arrest induced by vitexicarpin which indicated that mitotic arrest is independent of apoptosis.

Additionally, to determine the possible role of p53 in vitexicarpin-induced mitotic arrest, cells were treated with PFT-α, a specific inhibitor of p53 and cell cycle profile was analyzed by flow cytometry. We found that PFT-α treatment did not attenuate vitexicarpin mediated G2/M arrest in U87 cells. To further confirm, we observed the effect of vitexicarpin on cell cycle profile of H1299 (p53-/-) cells and found a similar cell cycle arrest at G2/M checkpoint in H1299 cell after exposure to vitexicarpin. Taken together, the data suggested that vitexicarpin induced mitotic arrest in U87 cells through inhibition of tubulin polymerization. Moreover, the cell cycle arrest is an early event in growth inhibition and is p53- and apoptosis-independent. However, it remains unclear whether apoptosis is dependent on cell cycle arrest or not.

In conclusion, our data demonstrated that vitexicarpin inhibited the growth of U87 glioblastoma cells by arresting the cell cycle at M phase through inhibition of tubulin polymerization and induction of apoptosis through mitochondrial caspase-3-dependent pathway.

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