WITHAFERIN A INDUCES APOPTOSIS IN RAT C6 GLIOMA CELLS THROUGH REGULATING NF-KB NUCLEAR TRANSLOCATION AND ACTIVATION OF CASPASE CASCADE

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Abstract

Background: The demand for the chemopreventive drug from the plant source is increasing in recent times, owing to its various biological activities without any adverse effect. The intention of this current study was to examine the anti-glioma effect of Withaferin A (WFA) on C6 glioma cell line model.
Materials and Methods: C6 glioma cells were administrated with different concentration of WFA (50, 100, 200 and 500 µg/mL) and DMSO (control) group to examine its anti-proliferative, anti-inflammatory and pro-apoptotic activities.
Results: Treatment with WFA showed a significant decline in the glioma cell count in a dose-dependent manner and thus proving its anti-proliferative effect. Similarly, inflammatory markers were also substantially lowered upon treatment with different concentration of WFA. However, DNA fragmentation and apoptotic markers like Caspase-3 and 9 were concomitantly enhanced after co-cultured with different concentration of WFA and thus exhibiting its cytotoxicity efficacy. Furthermore, the protein expression of Bcl2 and Bax were markedly downregulated and upregulated respectively; upon treatment with WFA on C6 glioma cells.
Conclusion: The outcome of this study evidently demonstrates that C6 glioma cells co-cultured with increased concentration of WFA, showed an anti-proliferative, anti-inflammatory and pro-apoptotic effect in a dose-dependent fashion.

Key words: Withaferin A, C6 glioma cells, anti-proliferative, anti-inflammatory, caspase, apoptosis

Introduction

Malignant glioma is one of the common and aggressive malignant tumors of central nervous system (neuroglial cells) with poor survival rate (Sciume et al., 2010). These glioma cells can rapidly proliferate with high invasive capacity which makes it one of the lethal malignances of neural system (Kiekow et al., 2016). Current therapies for malignant glioma are much limited since long term usage of those standard drugs or radiotherapy might lead to chronic cognitive impairment and life-threatening problems. In addition, the pathogenesis of glioma remains complicated and elusive (Shah et al., 2009). However, rapid proliferation, inflammation (infiltration) and anti-apoptosis are the trademarks for the malignant glioma (Christofides et al., 2015; McFaeland et al., 2013). Therefore, the requirement for the novel chemotherapeutic drug which could inhibit the rapid proliferation, inflammation as well as enhanced apoptosis would be the better option.

In recent times, the use of plant-derived products like polyphenols, steroids, alkaloids, terpenoids and tannins for treating various cancer conditions are increasing enormously owing to its several biological properties as well as non-toxic to normal cells (Lee and Choi, 2016). Withaferin A (WFA) is one of the major bioactive components of plant *Withania somifera* (Indian Ginseng) which is a popular Ayurvedic medicine and used for treating various disorders (Singh et al., 2011). WFA is a highly oxygenated withanolides (C28-steroidal lactone), with four cycloalkanes, one cyclopentane, and three cyclohexane rings. WFA exert a broad range of pharmacological properties such as antioxidant, anti-inflammatory, antiangiogenic, anti-tumor, anti-microbial and pro-apoptotic due to its alkylation and acylation of biomolecules especially proteins (Vyas and Singh, 2014; Berghe et al., 2012). Handful evidence inferred that WFA can interact with numerous proteins and thereby regulate various signaling pathways especially related to inflammation, proliferation and apoptosis (Heyninck et al., 2016). Previous studies also indicated that WFA can exhibit antitumor or anticancer properties against several types of cancer including breast, liver, breast, prostrate, head and neck cancer (Samadi et al., 2012; Grogan et al., 2013; Vel Szic et al., 2014). However, the chemoprotective effect of WFA against the malignant glioma on C6 glioma cell line was much limited, especially the correlation between inflammation and

apoptosis were not explored till date. Moreover, WFA has been reported to cross blood brain barrier (McFaeland et al., 2013) and hence it would act as an effective anti-glioma agent.

Because WFA can act as anti-proliferative (cytotoxic), the anti-inflammatory, anti-adhesive, anti-angiogenic and pro-apoptotic effect in many cancer cell line model (Vyas and Singh, 2014; Berghe et al., 2012) and thus it can be used as an anti-glioma agent. To prove the above statement, we designed our present study to evaluate the chemoprotective effect (anti-glioma) of WFA by examining the anti-proliferative, anti-inflammatory and pro-apoptotic activity on C6 glioma cell line model.

Materials and Methods
Chemicals

Withaferin A, dimethyl sulfoxide (DMSO), trypsin, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT), ethylene-diamine-tetraacetic acid (EDTA), glutamine, cell lysis buffer, isopropanol, and formalin were purchased from Sigma-Aldrich (MO, USA). Dulbecco's modified eagle's medium (DMEM), Fetal bovine serum (FBS), Penicillin and streptomycin, were bought from Thermo Fisher Scientific Gibco (NY, USA). All the other solvents and reagents used in the present study were of analytical grade.

Cell Culture

The C6 rat glioma cells were provided by the American Type Culture Collection (MD, USA). C6 glioma cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) added with 10% fetal bovine serum, 5 mM glutamine, 1% penicillin, streptomycin and HAT supplement (Gibco, NY, USA) in 5% CO_2 at 37°C. The media were changed twice a week. When C6 glioma cells reached confluence, they were subcultured with 0.25% trypsin/ 0.53 mM EDTA (trypsinization) and plated in 96-well cell culture plates.

MTT Assay

The cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) by the method of Liu *et al*. (2012). Briefly, an aliquot of 100 µL (2×10^5/mL) of C6 glioma cells was seeded in each well of the 96-well plate and incubated for 8-10 h at room temperature. Then, the media were replaced and treated with increasing concentrations of WFA (50, 100, 200 and 500 µg/mL) and incubated at 37°C for 24, 48 and 72 h. However, the control group wells are treated only with medium (without WFA with 0.2 % DMSO). At the end of each incubation time (24, 48 and 72 h), the media was replaced with fresh medium, containing 0.5 mg/mL MTT in phosphate buffered saline (PBS) and cultured at 37°C with 5% CO_2 for 4 h. Finally, 100 µL of isopropanol were mixed to each well and shaken well for 20 min. The absorbance (optical density) at 570 nm was determined using ELISA plate reader (Model-680, Bio-RAD; CA, USA) after 5 min to check the purple-blue MTT formazan crystals. The net absorbance indicates the cell viability. The cell viability (%) was plotted against the concentration of the WFA and calculated by the below formula,

Cell viability (%) = (1- A_{570} for WFA treated cells /A_{570}nm for control untreated cells) x 100%

DNA Fragmentation

Cellular DNA fragmentation (cell death detection ELISA plus) commercial ELISA kit from Roche Molecular Biochemicals (Mannheim, Germany) was used to measure the extent of C6 glioma cell DNA fragmentation. Briefly, C6 glioma cells (1×10^5/mL) were seeded in Petri dish (10 cm) and treated with different concentration of WFA for 48 h (control group with 0.2% DMSO). Then the cells were harvested and lysed using lysis buffer and centrifuged at 500 xg for 10 min at 4°C. Then, add 20 µL of supernatant containing cytoplasmic histone-associated DNA fragments and transferred to the immobilized anti-histone antibody coated plate and kit reagents were added (based on supplier protocol). The absorbance at 405 nm was measured using microplate reader. DNA fragmentation was calculated by the formula (enrichment factor).

DNA fragmentation = A_{405} for WFA treated cells /A_{405} nm for control untreated cells

Inflammatory Markers

The cell suspension was prepared as indicated in DNA fragmentation section. The NF-κB free p65 subunit in the nuclear fraction of lysed C6 glioma cells was separated by Nuclear/Cytosolic Fractionation Kit (Cell Biolabs Inc, SD, USA) and subsequently evaluated using an ActivELISA kit (Imgenex, CA, USA). Moreover, the levels of Tumor necrosis factor-α (TNF-α) were measured by ELISA using Quantikine TNF-α commercial kits (R&D Systems Inc, MN, USA) using a cytosolic fraction of C6 glioma cells by following the manufacturer's guidelines.

Apoptotic Markers

The activities of Caspase-3 and 9 were assessed by a colorimetric Caspase assay kit provided by Hushang Inc., (Shangai, China) by supplier protocol. Briefly, C6 glioma cells (1×10^5/mL) were seeded in petri dish and treated with different concentration of WFA for 24 h (control group without treatment). Cells were harvested and disintegrated by adding 50 mL of cell lysis buffer and centrifuged at 1500 xg for 10 min at 4˚C, then debris was removed. The total protein was extracted using a Nuclear/Cytosol Extraction Kit from Cell Biolabs Inc., (SD, USA) based on the manufacturer's specification; the protein contents were quantified by BCA Protein assay reagent kit (Pierce, IL, USA). An equal amount of proteins (50 µg) from each treatment group (50-500 ug/mL) were added to the reaction mixture (caspase substrate) and incubated for 1 h at 37˚C. The luminescence of each group was measured at 405 nm using a microplate reader. The values are expressed as the percentage of absorbance of treated group compared to the untreated control group (Control group was designed as 100%).

Western Immunoblot

The total protein extract for Bcl-2 and Bax quantification was prepared as indicated above and the equal volume of proteins (40 µg per lane) was resolved on 10% sodium dodecyl sulfate-polyacrylamide gel and then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Sigma; MO, USA). The membrane was blocked with TBS (Tris-buffered saline) which containing 0.3% Tween 20, 5% nonfat milk and 150 mM sodium chloride were incubated with primary antibody including mouse anti-Bcl-2 antibody, anti-Bax antibody (1:500; Santa Cruz Biotechnology, CA, USA) or rabbit monoclonal anti-rat β-actin (1:500; Zhongshan Biotechnology, Beijing) at 4°C for overnight and then washed with TBS to remove unbound antibodies. A secondary antibody was probed with horseradish peroxidase (HRP) linked anti-mouse antibody (1:1000; Santa Cruz Biotechnology, CA, USA) in TBS at room temperature for 1h and washed with TBS. The conjugated antibodies were detected by the enhanced chemiluminescence (Pierce; IL, USA) system, and the protein expression (band) are quantified using Bio-Rad image analyzer (GS-700 digital densitometer, GMI, Ramsey, MN 55303, USA). β-actin will serve as loading control.

Statistical Analysis

Results were reported as the mean ± standard deviation (SD). Each experiment was analyzed in triplicate. The difference between the treatment groups and control (DMSO) was examined by Student t-test using Statistical Package for the Social Sciences software (Version 23; IBM, NY, USA). A probability value of p < 0.05 was regarded as statistically significant.

Results and Discussion

To investigate the cytotoxicity (viability) of different concentration of WFA (0-500 µg/mL) in various duration (24, 48 and 72 h) on C6 glioma cells was assessed by MTT assay (Figure. 1). The IC_{50} viability rate for WFA was calculated to be 355 µg/mL for 24 h, 230 µg/mL for 48h and 150 µg/mL for 72 h, respectively. The outcome of MTT assay deduced that the C6 glioma cell count was significantly reduced upon treatment with WFA in a time-dependent manner and thus revealing its strong cytotoxic or anti-proliferative effect. Choudhary and his coworkers (2010) also indicated that treatment with different concentration of withaferin A on NCI-H460 human lung cancer cells showed a substantial decline in the number of viable cells and thus proved its cytotoxic activity. Also, Pretorious et al. (2009) indicated that Withaferin A and Withanolide-D present in *Withania somnifera* can induce cell death in MRC-5 cell model.

To evaluate whether WFA exhibit anti-proliferative activity (cell death-apoptosis) on C6 glioma cells, the levels of DNA fragmentation were determined using commercial ELISA kit method. As shown in figure 2, the levels of DNA fragmentation in control (DMSO treated) glioma cells are minimal. However, C6 cells co-cultured with increasing concentration of WFA showed a significant escalation in the levels of DNA fragmentation, when compared to control cells. The maximum concentration of WFA (500 µg/mL), demonstrated the highest DNA fragmentation levels as equivalent with other concentration. Our results are in correspondence with the results of Hahm et al. (2011), who demonstrated that withaferin A can trigger ROS generation and thus enhance DNA fragmentation (DAPI assay) and apoptosis. The results of MTT assay and DNA fragmentation were consistent with each other and thus demonstrate that WFA can induce cell death via apoptosis in a dose-dependent fashion. We speculate that WFA can significantly downregulate PI3K/Akt/mTOR signaling pathway and thereby suppress proliferation rate of C6 glioma cells. Recently, Grogan and others (2013) demonstrated that withaferin A can halt glioblastoma proliferation (cytotoxicity effect) via altering Akt/mTOR signaling pathway.

An impressing number of studies indicated that inflammation is one of the hallmarks for malignant glioma (Sciume et al., 2010; McFaeland et al., 2013). During malignant glioma condition, neuroglial cells and neutrophils are highly activated (infiltration) and contribute to the inflammatory response. Table 1 represents the effect of WFA on inflammatory markers on C6 glioma cells. C6 glial cells (control cells-DMSO) showed increased inflammatory

markers especially TNF-α and NF-p65 due to increased activation of neuroglial cells and neutrophils which may initiate the inflammatory cascade by triggering NF-κB and subsequently increasing various chemokines/cytokines such as TNF-α, IL-6 which in turn enhance the proliferation rate (Christofides et al., 2015). Whereas, co-cultured C6 glioma cells with WFA (50-500 µg/mL) concomitantly suppressed the levels of TNF-α and significantly inhibited the nuclear translocation of the NF-p65 subunit (from cytosol to nucleus). Our results are in congruent with the results of Martorana and his colleagues (2015), who also inferred that withaferin A can inhibit translocation of NF-p65 and thereby inactivate the production of proinflammatory cytokines like TNF-α. Furthermore, WFA has been proved to suppress the production of TNF-α via inactivation of NF-κB in endothelial cell line model (Lee et al., 2012).

Apoptosis is a programmed cell death, which is controlled by either pro-apoptotic factors (Bax and Bad) or anti-apoptotic factors (Bcl-2 and xl). Nevertheless, Bax and Bcl-2 are the major factors that regulate the apoptosis, since Bax can activate or inhibit Bxl and Bad, and also Bcl2 can inhibit Bax. Thus, for the present study, we concentrated only on Bax and Bcl2 to determine the rate of apoptosis. Also, these apoptotic factors can regulate the caspase cascade to execute the apoptosis process. Caspases (cysteine-aspartic proteases) are the family of the enzyme (protease) that plays an integral part in activating apoptosis. Those caspases were activated by the elevated amount of Bax (increased Bcl2/Bax ratio) and trigger the release of cytochrome c from mitochondria to cytosol. Then, cytochrome c can bind to Apaf-1 (apoptotic protease activating factor) and further activate the caspases like caspase 3 (effector), 9 (initiator) and thus subsequently lead to apoptosis (Frejlich et al., 2013). Hence, to determine, the effect of WFA on apoptosis, the caspase-3 and 9 levels (apoptotic marker) are evaluated to check the levels of apoptosis on C6 glioma cell line.

Caspase-3 and 9activity were exponentially elevated on C6 cells treated with increased concentration of WFA, as compared with untreated C6 cells (table 2). Similarly, Mandal and his colleagues (2008) demonstrated that treatment of withaferin A significantly increased (activated) the Caspase-3 and 9activity in leukemia cell line model. These findings manifest that WFA can be able to upregulate the activity of both the initiator and effector caspases 9 and 3 and thereby confirming the pro-apoptotic activity of WFA. Few studies also indicated that WFA can act as pro-oxidant and thus trigger oxidative stress to induce apoptosis (Vyas and Singh, 2014; Li et al., 2015).

As indicated earlier, apoptosis is initiated by either pro or anti-apoptotic factor. Out of those factors, Bax and Bcl2 play a pivotal factor in triggering apoptosis. The protein expression pattern of Bax and Bcl2 was tested by immunoblot technique (figure 3). The protein expression of Bax and Bcl2 was notably upregulated and downregulated, respectively on C6 glioma cells upon treatment with different concentration of WFA in dose gradient manner as compared with untreated C6 glioma cells. Therefore, we proved that WFA could effective trigger apoptosis and confirming its pro-apoptotic activity. Our results are in correlation with the outcome of Mayola and his colleagues (2011). Also, we evaluated the protein expression of nuclear NF-p65 subunits; it clearly depicts that nuclear NF-p65 subunit (active form) were considerably downregulated than DMSO-treated C6 glioma cells (data not shown). Hence, we concluded that by lowering NF-κB activation (NF-p65), WFA can downregulate Bcl2 (anti-apoptotic) and thereby upregulating Bax (apoptotic protein) which in turn elicit apoptosis via activating caspase cascade. Similarly, Kiekow et al. (2016) also pointed out that inactivation or inhibition of NF-p65 might downregulate Bcl2 family proteins which in turn activates the caspase cascade on C6 glioma cells and leads to apoptosis.

Few preclinical studies with a higher dosage (1gm/kg) of WFA did not trigger apoptosis (cell death) in healthy cells (Lee and Choi et al., 2016), whereas in this case (C6 glioma cells), WFA trigger apoptosis. From the above statement, it clearly illustrates that WFA probably triggers apoptosis only in a cancer cell, but not in healthy cells. Current experiment has few limitations such as, non-inclusion of flow cytometry to check the cell cycle inhibition activity if WFA as well as exclusion of invasive and metastasis parameters (extracellular matrix) to confirm the anti-glioma effect of WFA. Since it is a pilot study, we cannot include those parameters and techniques. However, in future studies, we will eradicate those limitations by including those parameters.
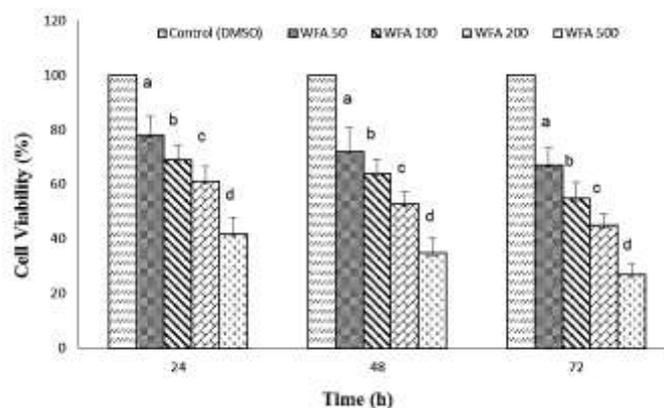
Figure 1: Effect of WFA on cell viability on C6 glioma cells by MTT assay. Values are expressed as the means ± SD. Data bearing different letters were significantly different ($p<0.05$).
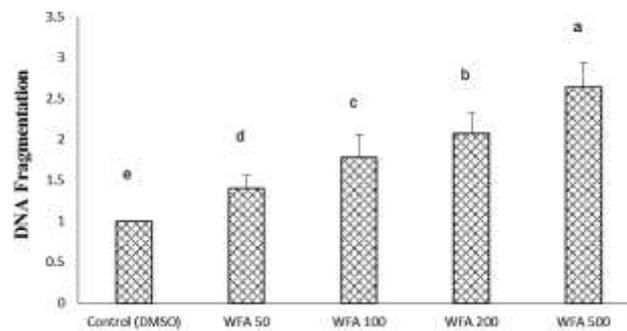
Figure 2: Effect of WFA on DNA fragmentation on C6 glioma cells. Values are expressed as the means ± SD. Data bearing different letters were significantly different ($p<0.05$).
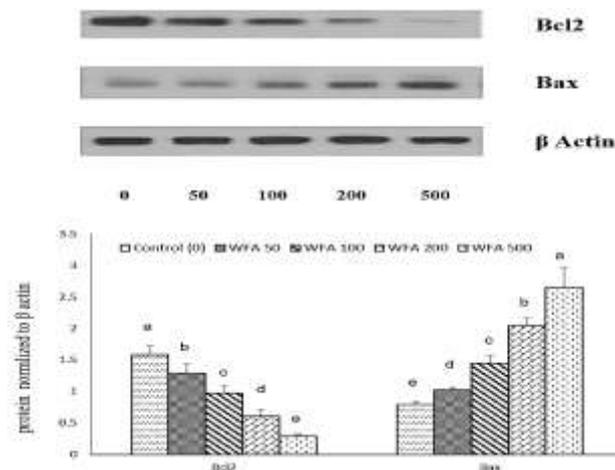
Figure 3: Effect of WFA on protein expression of Bcl2 and Bax on C6 glioma cells by immune blot technique. Values are expressed as the means ± SD. Data bearing different letters were significantly different ($p<0.05$).

Conclusion

Overall, the anti-glioma activity of WFA was well supported by our findings through inhibiting C6 proliferation (cytotoxic/anti-proliferative) and inflammatory response (NF-p65 translocation) and thus triggering apoptosis (activating caspase cascade) on C6 rat glioma cells. Further, the detailed mechanism for the anti-proliferative, anti-inflammatory and pro-apoptotic effect of WFA on C6 glioma cell lines have to be explored in future. Moreover, preclinical and are required to warrant the anti-glioma effect of WFA in malignant glioma animal model before it can be recommended for glioma patients.

Acknowledgements

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