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ORIGINAL ARTICLE

Multiflorane suppresses the proliferation, migration and invasion of human glioblastoma by targeting MAPK signalling pathway

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Summary

Purpose: *Glioblastoma is one common malignant tumors of central nervous system and its treatment is limited by the unavailability of chemotherapeutic agents. This study was therefore undertaken to evaluate the anticancer effects of Multiflorane molecule against the U87 human glioblastoma cells and normal human astrocytes.*

Methods: *The CCK8 assay was used to determine cell proliferation. Acridine orange (AO)/Ethidium bromide (EB) staining assays were used to detect apoptosis. Transwell assays were used to detect the cell migration and invasion. Western blotting was used to determine protein expression.*

Results: *The results showed Multiflorane inhibited the proliferation of the human U87 cells with little effects on the normal cells. Investigation of the underlying mechanisms showed that Multiflorane induced apoptosis in U87 cells. Multiflorane-induced apoptosis was linked with upregulation of cleaved caspase-3, 8 and 9, as well as cleaved PARP. The Bax protein levels were increased and of Bcl-2 were decreased. Flow cytometric analysis showed that Multiflorane induced G2/M arrest of the U87 glioblastoma cells. Transwell assays showed that the molecule suppressed the migration and invasion of the U87 glioblastoma cells. Additionally, Multiflorane also blocked the phosphorylation of p38 MAPK1 dose-dependently.*

Conclusion: *Taken together, Multiflorane may prove beneficial in the treatment of glioblastoma.*

Key words: *glioblastoma, apoptosis, cell cycle, multiflorane, proliferation*

Introduction

Being one of the ubiquitous types of plant secondary metabolites, triterpenoids have shown tremendous health promoting benefits [1]. Studies have shown that many of the triterpenoids suppress the growth of the human cancers [2]. The triterpenoids derived from plants have been used in the treatment of different human abnormal conditions in Asian countries [3].

Multiflorane has been isolated from several plant species such as *Cucurbita maxima* and *Trichosanthes kirilowii* to name a few [4,5]. Studies have shown that Multiflorane suppresses the growth of different types of cancer cells [6]. Glioblastoma, the most frequent type and malignant tumor of central nervous system, is responsible for tremendous human mortality [7]. The prognosis of glioblastoma patients is very poor and their average survival varies from 15 to 23 months. The 6% of 5-year survival is one of the worst among all cancer types. In 2017 approximately 0.79 million patients were expected to be diagnosed for glioblastoma [8]. Given this lethality, there is very much urgency to identify compounds that are safer and effective in the treatment of glioblastoma. The present study reported that Multiflorane significantly suppressed the growth of human glioblastoma cells via induc-

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tion of apoptosis and cell cycle arrest. This molecule also suppressed the migration and invasion of U87 glioblastoma cells via blocking of the MAPK signalling. Taken together, Multiflorane may prove beneficial in the glioblastoma treatment.

Methods

Growth and proliferation assay

Cell counting kit-8 (CCK-8, MedChemExpress, N.Jersey, USA) was used for estimation of the proliferative rates of glioblastoma cells treated with Multiflorane (96%, Sigma-Aldrich, St.Louis, Missouri, USA) which were compared with those of normal treated cells. In brief, the cells were placed in 96-well plates at 1×10^6 cells/well and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (St.Louis, Missouri, USA) for 24h with 0, 2.5, 5, 10, 20, 40, 80 and 160μM Multiflorane, after which CCK-8 was employed to estimate the proliferation rates by the addition of 10μl of CCK-8 solution to each well, at the indicated time intervals. Following 2 h incubation at 37°C, absorbance at 450 nm was read for each sample with a microplate reader.

Apoptosis assay

AO/EB staining followed to examine the effect of Multiflorane on the viability of glioblastoma cells and induction of cell apoptosis. The cells were plated into the 12-well plates at a density of 0.6×10^6 cells/well. Multiflorane at 0, 10, 20 and 40 μM concentrations was added to each well and cells were incubated with DMEM at 37°C for 24 h. Afterwards, the cells were harvested and washed twice with phosphate buffered saline (PBS) followed by fixing with 4% paraformaldehyde. The AO/EB solutions were then separately used to stain the cells. Afterwards, the cells were examined for the fluorescence measurements using fluorescent microscope. For the determination of the percentage of apoptosis Annexin V/PI staining was used as described elsewhere [2].

Cell cycle analysis

The U87 cells were harvested and washed twice with PBS. Cells were then fixed with 70% ethanol for about an h at -20°C and then washed again with PBS. Cells were resuspended in a solution of PI (50 μl/ml) and RNase1 (250 μg/ml) (Invitrogen Life Technologies, Massachusetts, USA). This was followed by incubation for 30 min at room temperature and fluorescence-activated cell sorting using 10,000 cells/group were assessed with a flow cytometer.

Migration and invasion assay

Transwell chambers with or without matrigel coating were used to assess the migration and invasion of transfected glioblastoma cells. Briefly, 100μl cell culture containing 6000 cells were added in the upper and lower chamber along with 750μl of DMEM medium supplemented with 10% fetal bovine serum (FBS). After 48-h incubation at 37°C/5%CO_2, cells from the surface of membrane's upper side were removed carefully with cotton swabs while those sticked to lower side of membrane were fixed with 70% ethyl alcohol and stained with 0.1% crystal violet. Light microscope (x100) was used for visualization of cells and photographs were taken. At least seven random fields were used for counting of migratory or invasive cells.

Western blotting

Using RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, Mass, USA), total proteins were isolated from untreated glioblastoma cells and cancer cells treated with 10, 20 and 40μM Multiflorane for 24 h. Bradford method was used to quantify the protein concentrations. About 45 μg of total proteins from each sample were separated electrophoretically on 10% SDS-PAGE. The gel was blotted to nitrocellulose membrane with primary protein antibodies overnight at 4°C. The blots were washed with TBS, incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc) for 1h at 4°C, washed again 3 times with TBS and chemiluminescence was captured on hyperfilm following incubation of blots in ECL plus solution followed by exposure of secondary antibodies. ECL was used for detection of bands corresponding to proteins of interest. The protein expression procedures were normalized with human GADPH protein.

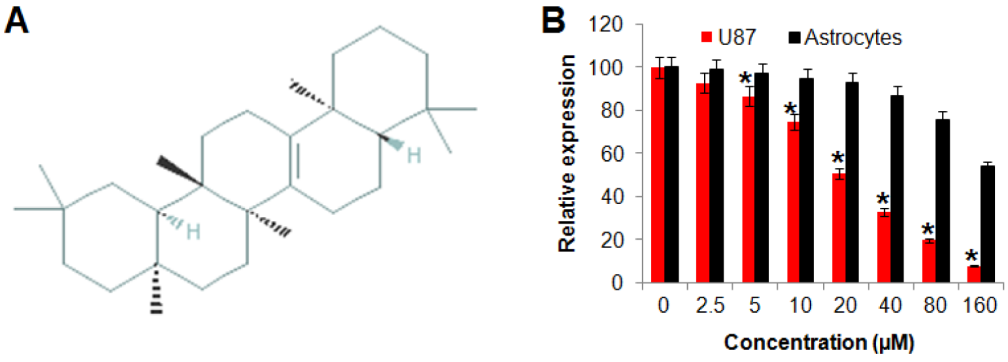

Figure 1. A: Chemical structure of Multiflorane. **B:** CCK-8 assay showing the effects of Multiflorane on the viability of the U87 glioblastoma and normal astrocytes. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Results

Inhibition of glioblastoma proliferation by Multiflorane

To ascertain the effects of Multiflorane (Figure 1A) on the proliferation of the glioblastoma, the normal astrocytes and U87 glioblastoma cells were treated with 0 to 160 μM concentrations of Multiflorane for 24 h. Using CCK-8 kit, the proliferation rates of cells were determined and found that the viability of U87 cancer cells decreased proportionally with increasing doses of Multiflorane with an IC_{50} of 20 μM (Figure 1B). Surprisingly, the effects of Multiflorane on the normal astrocytes were much less severe as evidenced from the IC_{50} of around 160 μM.

Multiflorane promotes apoptosis in U87 glioblastoma cells

The AO/EB staining showed that the growth inhibitory effects of Multiflorane on the glioblastoma cells were due to initation of apoptosis as evidenced from the increase in the orange colored U87 cells (Figure 2). The annexin V/PI staining showed that the percentage of the U87 glioblastoma cells increased significantly upon increasing concentrations of the molecule. The percentage of apoptosis increased from 4 to 71% from control to 40 μM Multiflorane (Figure 3).

Multiflorane alters apoptosis-related protein expression

The effect of Multiflorane was also assessed on the expression of the apoptosis-related proteins. The results showed a concentration-dependent increase in the proteins levels of cleaved caspsase-3, 8 and 9, as well as that of the cleaved PARP in Multiflorane-treated U87 cells. Additionally, the expression of Bax increased while as the proteins levels of Bcl-2 showed a dose-dependent decrease (Figure 4).

Multiflorane induces G2/M arrest of the U87 cells

The cell cycle analysis was performed to examine whether Multiflorane has any effects on the cell cycle distribution of the U87 cells. The results showed that upon treatment with Multiflorane the G2/M phase U87 cells increased concentration-

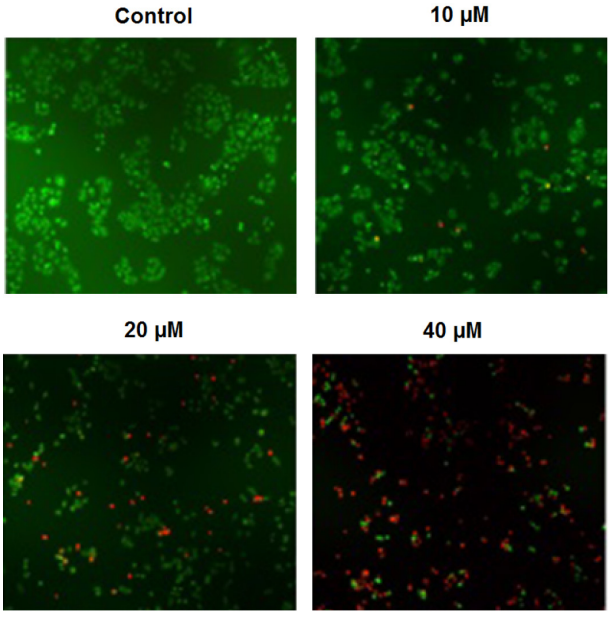

Figure 2. AO/EB staining assay showing Multiflorane induces apoptosis in U87 cells. The experiments were repeated in triplicate.

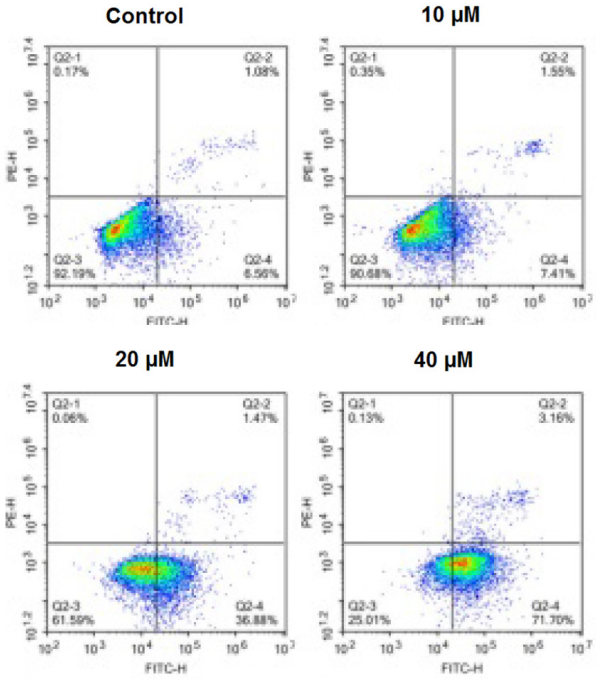

Figure 3. Annexin V/PI staining assay showing Multiflorane induces apoptosis in U87 cells. The experiments were repeated in triplicate.

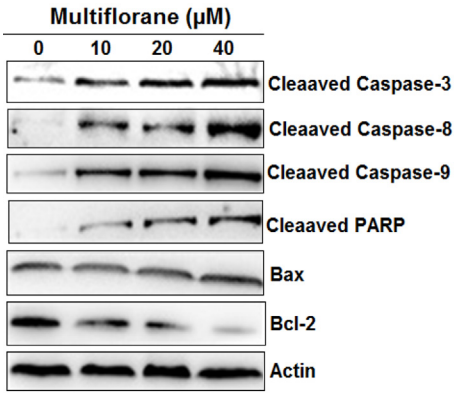

Figure 4. Western blots showing Multiflorane alters the expression of apoptosis-related proteins in U87 cells. The experiments were repeated in triplicate.

dependently. In comparison to 11% G2/M phase cells in control, the G2/M phase cells increased to 38% at 40 μM concentration of Multiflorane (Figure 5).

Suppression of migration and invasion of U87 cells by Multiflorane

The transwell assays were used to assess the effects of Multiflorane on the migration and invasion of U87 glioblastoma cells. The results showed that the molecule suppressed the migration of U87 cells concentration-dependently (Figure 6). The effects of Multiflorane were also determined on U87 cell invasion and the results were similar to cell migration (Figure 7).

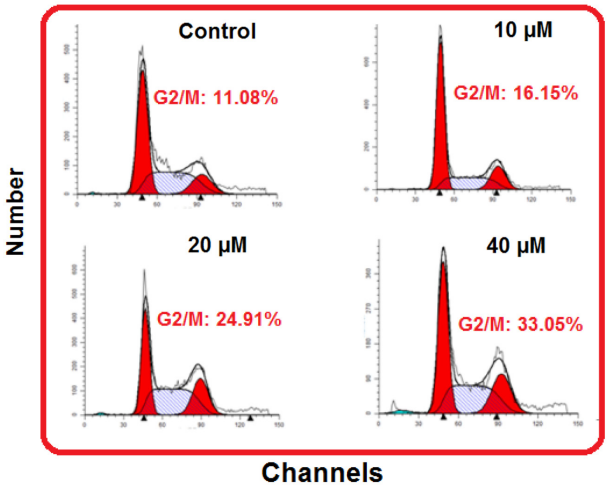

Figure 5. Flow cytometric analysis showing Multiflorane induces G2/M arrest in U87 cells. The experiments were repeated in triplicate.

Multiflorane MAPK1 signalling in U87 cells

The effects of Multiflorane were also evaluated on the MAPK1 pathway and the results showed concentration-dependent inhibition of phosphorylation of p38 (Figure 8). Nonetheless, there was no apparent effect on the total p38 levels.

Discussion

Triterpenoids constitute a large diverse group of plant secondary metabolites with enormous pharmacological potential [9]. They ubiquitously present across the plant kingdom. These plant-derived metabolites have a wide array of biological roles in plants which include defense against the biotic and abiotic stresses. A wide array of terpenoids have been reported to exhibit enormous pharmacological potential [10]. Accordingly, active research is going on to examine the anticancer effects of triterpenoids against different human cancers. This study was undertaken to evaluate the anticancer effects of Multiflorane, a naturally occurring triterpenoid of plant origin, against human glioblastoma cells. The cell proliferation assay showed significant inhibition of the glioblastoma cells growth upon Multiflorane treatment. Previous studies have shown that triterpenoids have the potential to trigger apoptosis in the glioblastoma and neuroblastoma cells [11]. Additionally, this study also showed that Multiflorane promoted the arrest of U87 cells at the G2/M check point of the cell cycle. This is consistent with a previous study wherein a triterpenoid Asiatic acid caused G2/M arrest of breast cancer cells [12]. The expression of

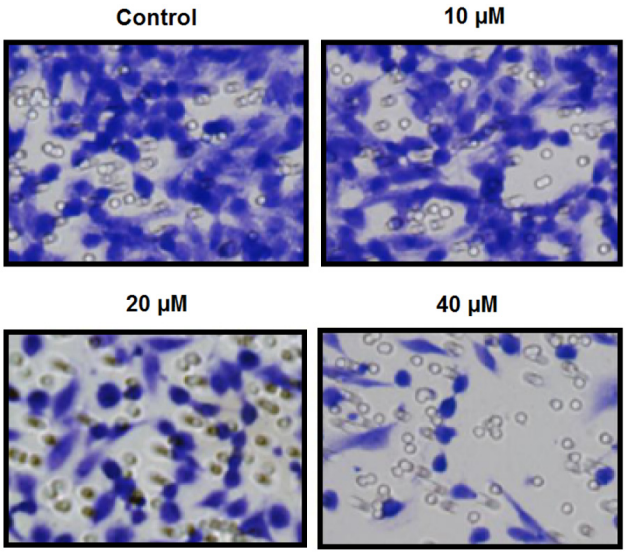

Figure 6. Transwell assays showing Multiflorane inhibits the migration of the U87 cells. The experiments were repeated in triplicate.

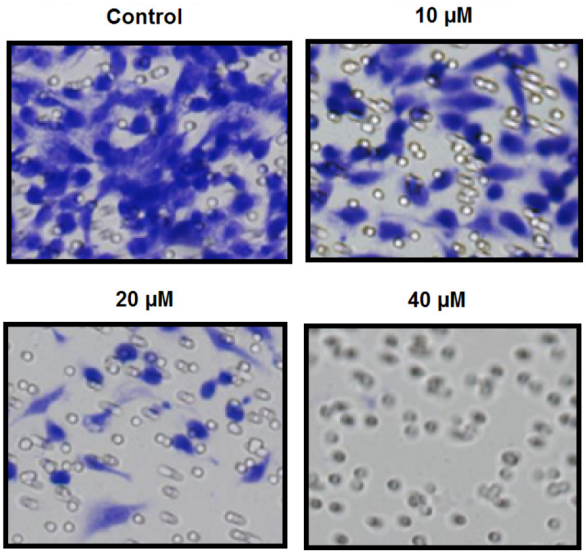

Figure 7. Transwell assays showing Multiflorane inhibits the invasion of the U87 cells. The experiments were repeated in triplicate.

cleaved caspase-3, 8 and 9 as well as that of cleaved PARP was remarkably increased. Additionally, the Bax/Bcl-2 ratio was also increased which is an important indicator of apoptosis [13]. Apoptosis plays key role in eliminating the defective cells and thus drugs that promote apoptosis are currently being studied extensively [14]. This study also examined the effects of Multiflorane on migration and invasion of the U87 cells and found that Multiflorane suppressed their migration and invasion. These findings are in agreement with a previous study wherein a triterpenoid Ursolic acid has been found to suppress the migration and invasion of cancer cells [15]. MAPK1 signalling is one of the critical signalling cascades that is dysregulated in cancer cells [16]. Herein we observed that Multiflorane blocks the MAPK1 signalling pathway. Hence more studies are required to establish Multiflorane as a lead molecule for the development of systemic therapy for glioblastoma.

Conclusion

Taken together, the results of the present study showed that Multiflorane inhibited the growth of the human glioblastoma cells via induction of apoptosis and G2/M cell cycle arrest. Multiflorane also suppresses the migration and invasion of the glioblastoma cells and may therefore prove beneficial in glioblastoma systemic treatment.

Conflict of interests

The authors declare no conflict of interests.

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