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ORIGINAL ARTICLE

Antitumor effects of Sweroside in human glioblastoma: its effects on mitochondrial mediated apoptosis, activation of different caspases, G0/G1 cell cycle arrest and targeting JNK/p38 MAPK signal pathways

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Summary

Purpose: *Glioblastoma is one of the prevalent types of brain tumors and is responsible for significant number of deaths world over. Glioblastoma is often diagnosed at advanced stages and there are frequent relapses following chemotherapy. Herein, we examined the anticancer effects of a secoiridoid glycoside Sweroside, against a panel of glioblastoma cells.*

Methods: *CCK8 assay was used to examine the anti-proliferative effects of this molecule. Acridine orange (AO)/ethidium bromide (EB) and annexin V/propidium iodide (PI) staining assays were used to examine apoptotic cell death. Cell cycle analysis was performed by flow cytometry. The protein expression was examined by western blotting.*

Results: *Sweroside inhibited the growth of the glioblastoma U251 cell with IC_{50} of 10 μM. However, Sweroside had low cytotoxic effects on the normal astrocytes cells with an IC_{50} of 100 μM. Sweroside exerted antiproliferative effects on the U251 glioblastoma cells by apoptotic cell death. This was concomitant with upregulation of apoptotic proteins such as caspase 3 and 9, and Bax expressions. Sweroside also induced arrest of the U251 cells at the G0/G1 phase of the cell cycle. Finally, Sweroside also blocked the JNK/p38 MAPK signal pathway concentration-dependently in U251 glioblastoma cells.*

Conclusions: *Taken together, these results suggest that Sweroside exerts potent anticancer effects on glioblastoma cells and may prove essential in the management of glioblastoma.*

Key words: *glioblastoma, sweroside, apoptosis, cell cycle arrest*

Introduction

Glioblastomas are prevalent malignancies of the nervous system, accounting for significant mortality throughout the world [1]. The prognosis of glioblastomas is generally dependent on the histological grade [2]. Given the low survival rate of high grade gliomas [3], there is urgent need to develop novel molecular targets and reliable chemotherapy. Natural products, especially plants, are rich sources of metabolites that can serve as drugs for the treatment of deadly human diseases, including cancer [4]. In order to combat the severe environmental stresses, plants have developed diverse ways to adapt themselves, over the course of evolution. One of the imperative mechanisms, is to synthesize a battery of chemicals, often referred as secondary metabolites [5]. Since, ancient times, the plant extracts rich in secondary metabolites have been used for treating human diseases. Some

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of the plant extracts are used as medicines in different traditional medicine systems even today. However, the advent of natural product chemistry allowed the isolation of the bioactive molecules from plants and their subsequent use as drugs in the purest form [6]. Sweroside is an important secondary metabolite belonging to the class of metabolites known as secoiridioids [7]. Sweroside is generally isolated from the species of Genus Swertia [8]. Although there is limited information on the bioactivity of chemical constituents of Swertia, the bioactivities of the Swertia extracts are well documented. These bioactivities include anti-oxidant, anti-hepatitis B virus, anti-microbial and anti-inflammatory to name a few [9]. Herein we examined the anticancer effects of Sweroside, an important secoirioidid found in Genus Swertia, against the human U251glioblastoma cells and attempted to explore the molecular mechanisms responsible for the anticancer effects of Sweroside. The main purpose of the current investigation was to study the anticancer effects of sweroside along with evaluating its effects on apoptosis, caspases, cell cycle arrest and JNK/p38/MAPK signal pathways.

Methods

Cell viability determination

The CCK-8 assay was used to monitor the proliferation of the normal human astrocytes and glioblastoma cells. In brief, the transfected U251 glioblastoma and normal human astrocytes cells were seeded in 96-well plates and subjected to treatment with varied concentrations of Sweroside at 37°C for 24 h. Thereafter, 10 µL of CCK-8 solution were added to the cell culture and incubated for 2 h at 37°C in a humidifier (5% CO_2, 95% O_2). Optical density $(OD)_{450}$ was assessed with microplate reader to determine the cell viability.

Detection of apoptosis

The U251 cells at the density of 0.6×10^6 were cultured in 6-well plates. Following an incubation period of around 12 h, the U251 cells were subjected to Sweroside treatment for 24 h at 37°C. As the cells were harvested, 25 µl cell cultures were put onto glass slides and stained with 1 µl solution of AO/EB. The slides were cover-slipped and examined with a fluorescent microscope. Annexin V/PI staining was performed as described previously [10].

Cell cycle analysis

The U251 cells were treated with varied concentrations of Sweroside and incubated for 24 h at 37°C. The cells were subjected to washing with phosphate buffered saline (PBS). Afterwards, the Sweroside-treated U251 cells were stained with PI and the distribution of the cells in the cell cycle phases was assessed by FACS flow cytometer.

Western blot analysis

Protein expression estimation was carried out by western blotting. The Sweroside-treated U251 cells were harvested with centrifugation and were then lysed in lysis buffer containing the protease inhibitor. About 45 µg of protein was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Afterwards, the membranes were treated overnight with primary antibodies at 4°C. Subsequently, the membranes were incubated with secondary antibodies. Finally, the signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalization.

Statistics

SPSS software was used for statistical analyses which were performed by Student's t-test. The experiments were carried in triplicate and the data are presented as mean ± SD. $P<0.05$ denoted statistical significance..

Results

Sweroside inhibits the proliferation of the U251 glioblastoma cells

To ascertain the anti-cancer effects of Sweroside, the U251 cells were treated with 0-100 µM Sweroside and then subjected to CCK8 assay. The outcomes of the CCK8 cell viability assay showed

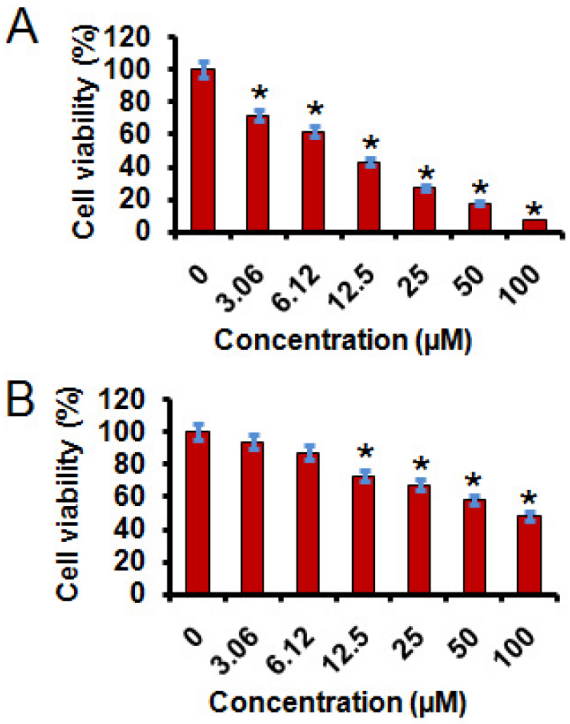

Figure 1. Effects of Sweroside on the **(A)** human glioblastoma U251 and **(B)** normal human astrocytes as determined by the cell viability assay. The experiments were performed in triplicate and expressed as mean ± SD (*$p<0.05$).

that Sweroside causes concentration-dependent decrease in the viability of the U251 cells (Figure 1A). It was further found that at 24 h of incubation, Sweroside exhibits IC_{50} of 10 μM against the U251 glioblastoma cells. However, Sweroside did not exhibit significant toxic effects on the normal human astrocytes as evidenced from the IC_{50} of 100 μM.

Sweroside induces apoptotic cell death of U251 cells

The apoptosis in the Sweroside-treated U251 cells was determined by AO/EB staining. The AO/EB staining revealed that Sweroside triggered apoptosis as evidenced from nuclear fragmentation of the Sweroside-treated U251 cells (Figure 2). Moreover, the results of the orange colored cells increased with increase in the concentration of Sweroside, indicative of apoptotic cell death. Annexin V/PI staining showed that the apoptotic U251 cell percentage increased to about 29.59% at 20 μM concentration of Sweroside as compared to approximately 3% in the untreated U251 cells (Figure 3). Furthermore, the expression of Bax was significantly enhanced while of Bcl-2 was decreased upon Sweroside treatment (Figure 4).

Sweroside effects the caspase expression in the U251 cells

Since Sweroside-induced apoptosis in the U251 glioblastoma cells, western blot analysis was performed to determine the expression of Caspase-3 and Caspase-9. The results showed that Sweroside caused a significant increase in the expression of Caspase-3 and 9. These effects of Sweroside on the U251 glioblastoma cells were concentration-dependent.

Sweroside impacts cell cycle distribution

The effects of Sweroside were also investigated on the cell cycle distribution of the U251 cells by flow cytometry. It was found that Sweroside caused significant increase in the percentage of the G0/G1 phase U251 cells. The percentage of the G2/M phase cells increased to 70.05% at 20 μM as compared to 42.22% in controls (Figure 6). The induc-

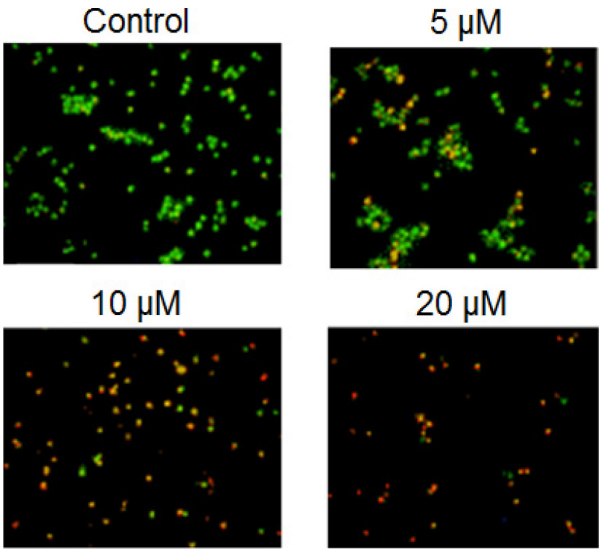

Figure 2. AO/EB staining showing that Sweroside induces concentration-dependent apoptosis in the human U251 glioblastoma cells. Red and yellow fluorescent cells indicate cells in different phases of apoptosis (early and late apoptosis), and it was observed that the percentage of apoptotic cells increased with increasing sweroside dose. The experiments were performed in triplicate.

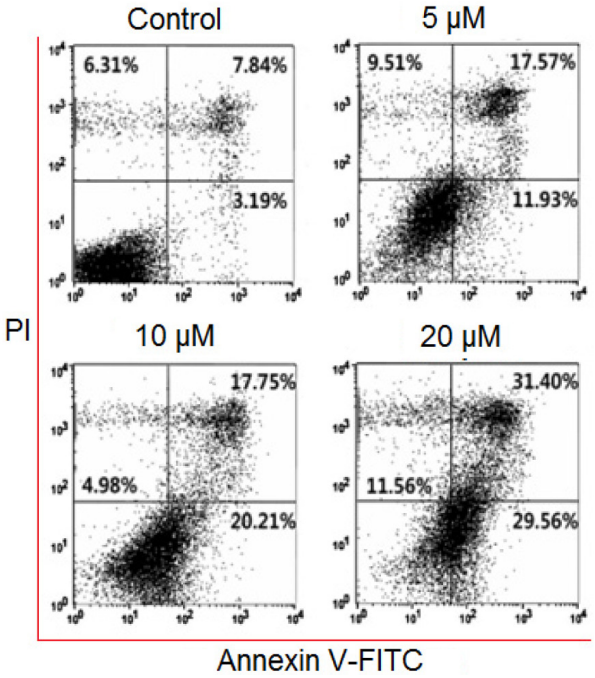

Figure 3. Annexin V/PI staining showing the effect of Sweroside on the percentage of the apoptotic of U251 cells. With increasing sweroside dose the percentage of apoptotic cells increased significantly. The experiments were performed in triplicate.

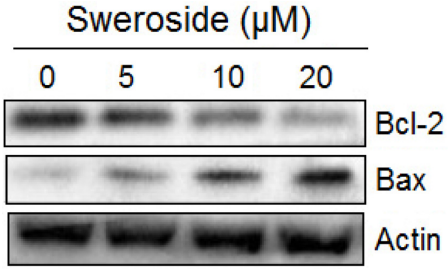

Figure 4. Effect of Sweroside on the expression of Bax and Bcl-2 as determined by western blot analysis. With increasing sweroside dose, the expression of Bcl-2 decreased while that of Bax increased in a dose-dependent manner. The experiments were performed in triplicate.

tion of the G0/G1 phase arrest by Sweroside in the U251 cells was also concomitant with concentration-dependent decrease in the expression of cyclin B1 (Figure 7).

Sweroside inhibits the JNK/MAPK signalling pathway in U251 cells

We also searched for the effects of Sweroside on the JNK/MAPK signalling pathway by western blot analysis at 0, 5, 10 and 20 μM concentrations. The results showed that Sweroside caused decrease in the expression of p-p-38 and p-JNK in a concentration-dependent manner (Figure 8). However, no visible effects were observed on the total JNK and p38.

Discussion

Glioblastoma causes significant mortality and morbidity across the globe and the need of the hour is to identify molecules, especially of plant origin, to combat this deadly disease [11]. Plant-derived molecules have shown exceptional potential to inhibit the growth and development of cancers [12]. They inhibit the proliferation of different mechanisms which include the induction of apoptosis, autophagy and arrest of cancer cells at different cell cycle phases [13]. Many of the plant-derived molecules have been shown to deactivate the signalling pathways that are generally activated in cancer cells. Alternatively they activate the signalling pathways that are generally deactivated in cancer cells [14]. Moreover, plant-derived molecules are believed to be safer for human consumption owing to their minimal adverse effects [15]. As such it is believed that anticancer drugs that are of plant origin may exhibit lower or even no side effects on the overall health of the cancer patients [15]. Herein, the anticancer effects of a plant-derived secoiridoid, Sweroside, were examined against the human U251 cells as well as the

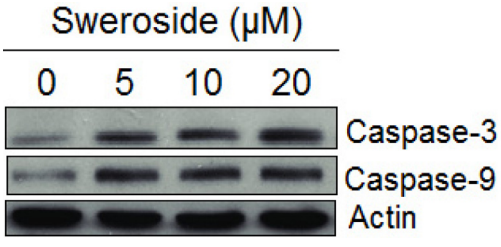

Figure 5. Effect of Sweroside on the expression of caspase-3 and caspase-9 as determined by the western blot analysis. The expression of both caspase-3 and -9 increased in a dose-dependent manner. The experiments were performed in triplicate.

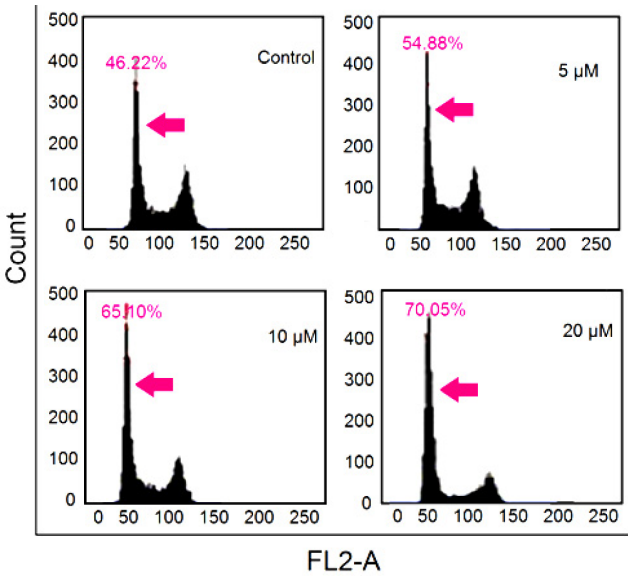

Figure 6. Effects of Sweroside on the distribution of the U251 cells in different cell cycle phases as determined by flow cytometry. The percentage of G0/G1 cells (as shown by arrows) increased in a dose-dependent manner from 46.22% in untreated control to 70.15% at 20μM dose. The experiments were performed in triplicate.

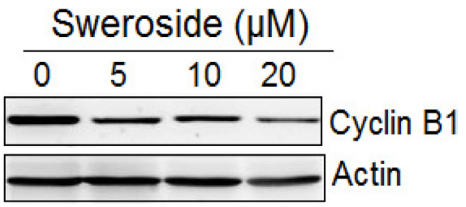

Figure 7. Effect of Sweroside on the expression of cyclin B1 as determined by the western blot analysis. The Figure shows that sweroside led to increase in the expression of cyclin-B1 dose-dependently. The experiments were performed in triplicate.

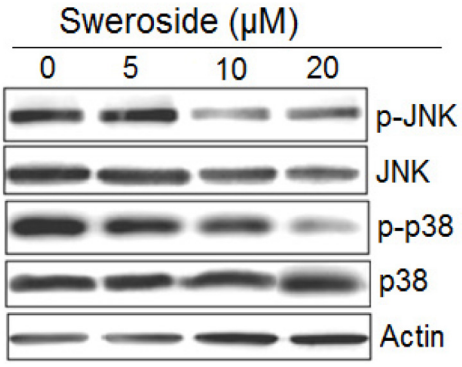

Figure 8. Effect of Sweroside on JNK/MAPK signalling pathway in U251 glioblastoma cells as determined by the western blot analysis. The Figure shows that sweroside caused decrease in the expression of p-p38 and p-JNK in a concentration-dependent manner. However, no visible effects were observed on the total JNK and p38. The experiments were performed in triplicate.

normal human astrocytes. The results showed that Sweroside inhibited dose-dependently the growth of cancer cells and exhibited an IC_{50} 10 μM. Nonetheless, it was interesting to see that Sweroside exhibits minimal growth inhibitory effects on the normal human astrocytes and exhibited an IC_{50} of 100, ie 10 times higher than that against the U251 cells. These observations suggest that Sweroside selectively targets the glioblastoma cells. Previous studies have shown that a number of plant-derived molecules inhibits the growth of cancer cells, for example, Isoalantolactone suppresses the proliferation of the UM-SCC-10A cancer cells by prompting apoptotic cell death [16]. Similarly, Quinacrine suppresses the growth of breast cancer cells by halting the topoisomerase activity [17]. To investigate the underlying mechanisms for the anticancer effects of Sweroside, AO/EB and Annexin V/PI staining assays were performed and both of these assays showed that Sweroside induces apoptosis and the percentage of the apoptotic cells increases with increase in the concentration of Sweroside. Bax and Bcl-2 are considered as vital biomarker proteins of apoptosis [18] and herein we observed that Sweroside caused increase in the expression of Bax and decrease in the Bcl-2 expression, ultimately favoring apoptosis. The induction of apoptosis has also been reported to be accompanied with the activation of different caspases [19] and herein we investigated the effects of Sweroside on the expression of caspase-3 and 9 and interestingly it was found that this molecule caused upregulation of both caspases concentration-dependently. Cell cycle arrest is another mechanism by which plant-derived anticancer agents have been reported to exert their anticancer effects [20]. Herein, we found that Sweroside caused arrest of the U251 cells in the G0/G1 checkpoint of the cell cycle. JNK/MAPK signalling cascade has been shown to be activated in cancer cells and believed to be responsible for the development and progression of different mechanisms [21]. What we found was that Sweroside blocks this pathway, suggestive of the potent anticancer effects of Sweroside.

Conclusion

The findings of the present study suggest Sweroside exerts significant anticancer effects on the human glioblastoma cells. The anticancer effects of Sweroside are mainly attributed to apoptosis induction and cell cycle arrest. Sweroside may be utilized in the development of systemic therapy for glioblastoma and deserves further studies.

Acknowledgements

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Conflict of interests

The authors declare no conflict of interests.

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