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ORIGINAL ARTICLE

Cycloartenol exerts anti-proliferative effects on Glioma U87 cells via induction of cell cycle arrest and p38 MAPK-mediated apoptosis

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Summary

Purpose: *Gliomas are destructive malignancies affecting mainly the central nervous system. Gliomas constitute around 50% of all the central nervous system tumors. The purpose of this study was to examine the anticancer activity of cycloartenol against the glioma U87 cells and to investigate the underlying mechanisms.*

Methods: *MTT and colony formation assay were used to determine the proliferation rate. Acridine orange/ethidium bromide (AO/EB) and annexin V/propidium iodide (PI) were used to determine apoptosis and cell cycle analysis was carried out by western blotting. Cell migration was checked by cell migration assay and immunoblotting was used for checking protein expressions.*

Results: *The results revealed that cycloartenol inhibited the proliferation and the colony formation potential of the glioma U87 cells in a concentration-dependent manner. The antiproliferative effects were found to be due to induction of Sub-G1 cell cycle arrest and triggering of apoptosis. These effects were found to be dose-dependent. Cycloartenol also caused significant alteration in the expression of Bax and Bcl-2. Furthermore, cycloartenol inhibited the migration of glioma cells and suppressed the phosphorylation of the p38 MAP kinase.*

Conclusions *These findings indicate that cycloartenol may prove beneficial in the treatment of glioma and warrants further investigation.*

Key words: *apoptosis, cell cycle arrest, cell migration, cycloartenol, glioma*

Introduction

Plants are often exposed to extreme environmental conditions and they biosynthesize a wide assortment of metabolites for their own protection. These metabolites have been utilized by mankind for the treatment of many diseases since decades [1]. There are different categories of metabolites and tritepenoids form one of the largest groups of plant secondary metabolites [2]. Triterpenoids have been shown to exhibit a wide array of activities such as anticancer and antimicrobial [3]. Cycloartenol is one of the important tritepenoids prevalently found in plants. Most of the plant-derived steroids are biosynthesized from cycloartenol [4]. Cycloartenol has been shown to possess several bioactivities. *Garcinia kola* extract containing cycloartenol has been shown to exhibit broad spectrum antimicrobial activity [5]. Moreover, many tritepenoids such as ursolic acid, have been reported to exhibit anticancer effects against a wide range of cancer types [6,7]. However, the anticancer potential of

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cycloartenol has not been sufficiently studied, especially against glioma. The present study was carried out to study the effects of cycloartenol against the glioma U87 cells.

Gliomas are destructive malignancies which have been reported to affect mainly the central nervous system. Gliomas constitute around 50% of all the central nervous system tumors and cause significant mortality, e.g. patients with advanced gliomas survive for a mean of 16 months only [8].

Constant relapses and the side effects of the drugs obstruct proper treatment of gliomas [9]. In this study the anticancer effects of cycloarterenol against the glioma U87 cells were evaluated and the undellying mechanism was explored.

Methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich Co, USA. The antibodies were procured from Santa Cruz Biotechnology Inc (Dallas, Texas). The fluorescent probes 4'-6-diamidino-2-phenylindole (DAPI), PI, fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine and antibiotics were procured from Invitrogen Life Technologies (Jinan, Shandong, China).

Cell line and culture conditions

The glioma U87 cell line and normal human astrocytes were procured from Cancer Research Institute of Beijing, China, and cultured continuously in RPMI-1640 supplemented with 10% FBS containing antibiotics [streptomycin (100 µg/ml)) and penicillin G (100 U/ml)], and incubated at 37°C in an atmosphere containing 5% CO_2.

MTT assay

The impact of cycloartenol on the proliferation rate of U87 glioma cells was investigated using MTT assay. The cells (1x10^6 cells per well) were cultured in 96 well plates for 12 hrs and were exposed to 0-100 µM cycloartenol for 24 hrs and afterwards MTT solution (20 µl) was added. Before the addition of 500µl of DMSO for solubilizing the formazan crystals, dimethylsulfoxide (DMSO,500 µL) was added. Absorbance was thereafter read in an ELISA plate reader at 570 nm.

Clonogenic assay

To assess the impact of cycloartenol on the colony development capacity of U87cell line, the cells were collected at the exponential phase of growth and counted using a hemocytometer. The platting of the cells was carried out at 200 cells in each well. The plates were then kept for 48 hrs at 37°C to allow adhesion. This was followed by the addition of various concentrations (0, 20, 40 and 80 µM) of cycloartenol. The plates were again subjected to incubation for 6 days, after which they were subjected to washing with FBS and fixed with methanol. The U87 cells were then stained with crystal violet, followed by microscopy, and counted under light microscope.

Detection of apoptosis by AO/EB and annexin V/PI staining

U87 cells were grown in 6-well plates at 1×10^6 cells per well and then treated with 0, 20, 40 and 80 µM cycloartenol for 24 hrs. This was immediately followed by AO/EB staining. The cells were examined and photomicrographs were taken via fluorescence microscope. Thereafter, the cells were harvested, washed with FBS, and then treated with Annexin V/FITC and PI for 20 min. The percentage of apoptotic cells was checked by flow cytometry.

Determination of cell cycle phase distribution of U87 cells

Approximately 1x10^5 cells in each well in 6-well plates were kept at 37°C for 24 hrs to allow the cells to adhere and subsequently treated with various doses of cycloartenol. The cells were then subjected to incubation for 24 hrs at 37°C. The distribution of the U87 cells in various cell cycle phases was determined by flow cytometry.

Transwell cell migration assay

The U87 cells were suspended in 2% FBS and placed in the upper chamber with transwells of 8µm pore size. The medium was subsequently supplemented with 10% FBS and the cells were subjected to 24 h-incubation at 37°C. The un-migrated U87 cells were removed from the upper side of the membrane, while migrated cells on the lower surface were subjected to fixation with 100% methanol followed by Giemsa staining. Cell migration was determined by estimating the number of the migrated cells, using microscopy.

Western blotting analysis

The glioma U87 cells were lysed with the help of the ice-cold hypotonic buffer. After estimating the protein concentrations in each of the cell extracts, the samples containing the proteins were loaded and separated on SDS–PAGE. This was followed by transfering to a nitrocellulose membrane and incubation with the primary antibody (1:1000) for 24 hrs at 4 °C. Thereafter, the membrane was incubated with HRP-conjugated secondary antibody (1:1000) for at 24°C for about 1 hr. The visualisation of the proteins was carried out by enhanced chemi-luminescence reagent.

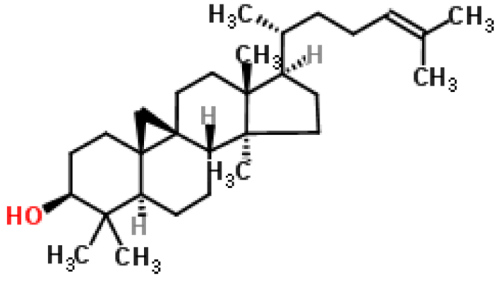

Figure 1. Chemical structure of cycloartenol.

Statistics

Data are presented as mean ± SD and were statistically analyzed using Student's *t*-test. GraphPad 6 software was used to perform the analyses. The levels of significance were determined at $p < 0.05$.

Results

Cycloartenol exerted anticancer effects on glioma U87 cells

After evaluating the antiproliferative effects of cycloartenol (Figure 1) on the glioma U87 cells, it was observed that the compound inhibited the proliferation of the glioma U87 cells. Cycloartenol exhibited an IC_{50} of 40 μM against the glioma U87 cells. Nonetheless, it showed lower growth inhibitory activity on normal human astrocytes (IC_{50} of 85 μM). The effect of cycloartenol on the growth of U87 cells followed a concentration-dependent pattern (Figure 2). It was also observed that cycloartenol treatment caused significant reduction in U87 colonies dose-dependently (Figure 3).

Cycloartenol triggered apoptosis in glioma U87 cells

Cycloartenol caused apoptotic cell death in U87 cells dose-dependently as indicated by AO/EB staining (Figure 4). The analysis of apoptotic cell population was performed via flow cytometry after

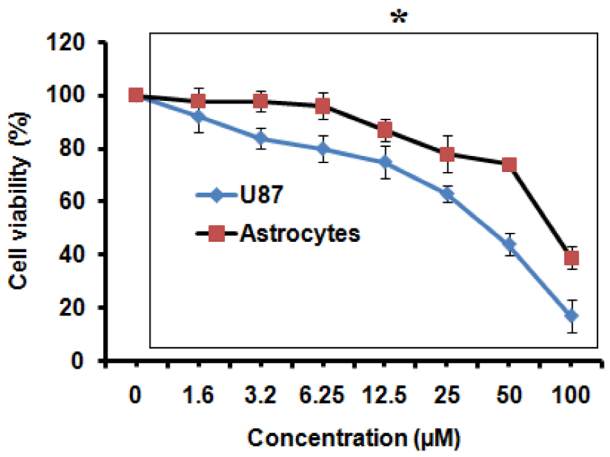

Figure 2. Cycloartenol inhibits the proliferation of human glioma U87 cells as determined by MTT assay. The experiments were repeated in triplicate and shown as mean ± SD (*p<0.05).

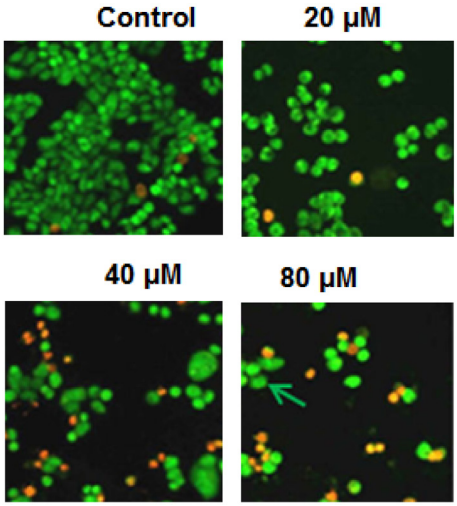

Figure 4. Cycloartenol triggers apoptosis in glioma U87 cells concentration-dependently as shown by the AO/EB staining. The experiments were carried out in triplicate. Arrow shows apoptotic cells.

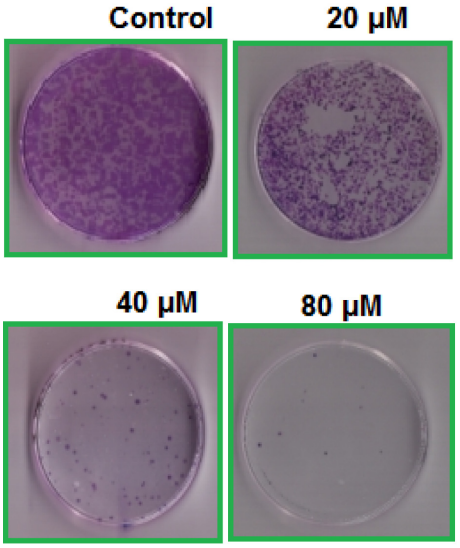

Figure 3. Cycloartenol inhibits the colony formation potential of human glioma U87 cells concentration-dependently, as determined by MTT assay. The experiments were carried out in triplicate.

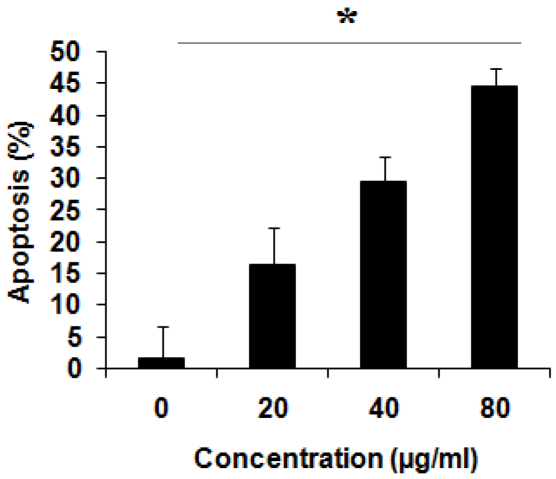

Figure 5. Determination of percent apoptotic cells at indicated concentrations of cycloartenol. The experiments were carried out in triplicate and show that the percent of apoptotic cells increase with increase in the concentration of cycloarterenol (*p<0.01).

annexin V/PI staining (Figure 5). The apoptotic U87 cells increased from 1.76% in the control to 44.58% at 80 μM of cycloartenol. The protein expressions of Bax and Bcl-2 were studied in cycloartenol-treated glioma U87 cells and showed that while the expression of Bax was increased in a dose-dependent manner, Bcl-2 expression decreased (Figure 6).

Cycloartenol triggered cell cycle arrest

For analysis of the distribution of the cycloartenol-treated glioma cells in different cell cycle phases, flow cytometery was performed. The results revealed that the percentage of the glioma cells increased significantly in the sub-G1 phase of the cell cycle. This effect of cycloartenol showed a dose-dependent pattern (Figure 7).

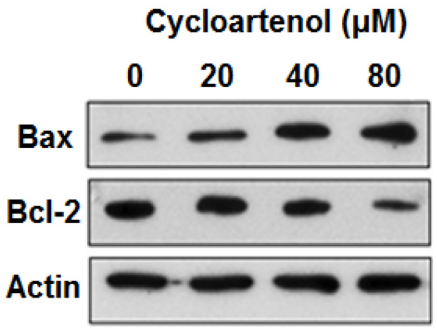

Figure 6. Effect of cycloartenol on the Bax and Bcl-2 expression in glioma U87 cells as shown by western blotting. The experiments were performed in triplicate and show that cycloarterenol inhibitd the expression of Bcl-2 and upregulates the expression of Bax in a concentration-dependent manner.

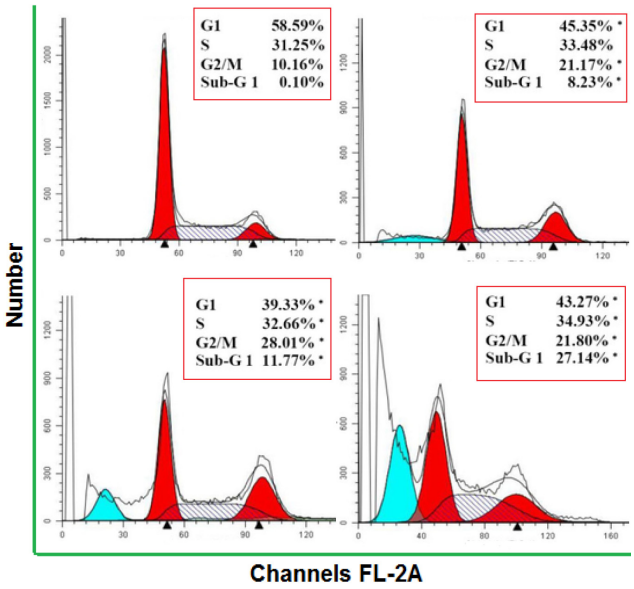

Figure 7. Cycloartenol induces sub-G1 cell cycle arrest in glioma U87 cells in a concentration-dependent manner, as shown by the AO/EB staining. The experiments were carried out in triplicate.

Cycloartenol inhibited the migration of the glioma U87 cells

Analysis of the effect of cycloartenol on the migration of the glioma U87 cells at 0, 20, 40 and 80 μM concentrations revealed that cycloartenol could inhibit the motility and the migration of the glioma U87 cells in a concentration-dependent manner (Figure 8).

Cycloartenol inhibited the p38 MAPK signalling pathway

The effect of cycloartenol was also investigated on the p38 MAPK signalling pathway. The results revealed that cycloartenol inhibited the expression of (phosphorylated) p-p-38 in a concentration-dependent manner. However, the expression of p-38 increased as depicted in the western blot (Figure 9).

Discussion

Gliomas are malignancies with very poor prognosis and tremendous mortality throughout the world [10]. Constant relapses and development

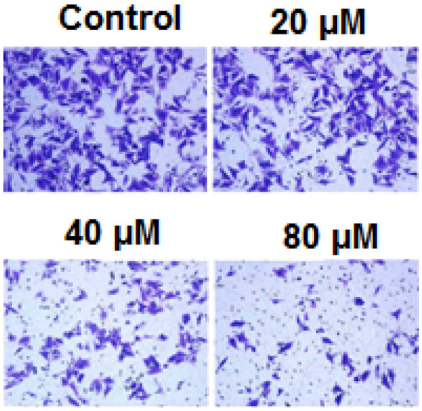

Figure 8. Cycloartenol inhibits the migration of glioma U87 cells in a concentration-dependent manner, as shown by the AO/EB staining. The experiments were carried out in triplicate.

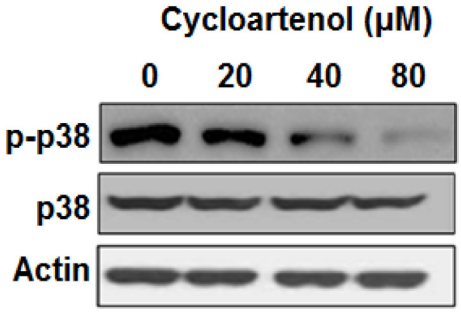

Figure 9. Cycloartenol inhibits the p-p38 MAPK signalling pathway in glioma U87 cells concentration-dependently, as indicated by western blotting. The experiments were carried out in triplicate.

of chemoresistance form the main obstacles in the treatment of gliomas [11]. Additionally, a number of side effects of the synthesized anticancer drugs exerts significant adverse effects [12]. These factors have created the need to look for safe plant-derived anticancer agents. The natural products from plants and microbes have played a significant role in the development of chemotherapy for the treatment of cancer [13]. These natural sources still exhibit tremendous potential as sources of drugs [14]. Plant-derived tritepenoids are being considered important drug candidates and several plant-derived tritepenoids have been reported to exhibit anticancer activity [15]. In the present study it was shown that cycloartenol inhibits the proliferation of glioma U87 cells as indicated by the findings of MTT assay. These results are also complemented by the colony formation assay wherein it was found that cycloartenol could suppress the colony formation by glioma U87 cells. Several other studies carried out previously have also shown that tritepenoids, such as ursolic acid, inhibit the proliferation of cancer cells [14]. Apoptosis is a vital mechanism which permits the body to eliminate the cancer cells from the body. As far as cancer is concerned, apoptosis also prevents the development of chemoresistance in cancer cells [6,7]. As such molecules that can prompt apoptosis in cancer cells are considered important candidates for the development of systemic cancer therapy. The results of AO/EB and annexin V/PI staining revealed that cycloartenol induces apoptotic cell death of the glioma U87 cells. This was further confirmed by the alteration in the Bax/Bcl-2 ratio which is an important determinant of apoptosis. Furthermore, cancer cells divide rapidly and invade other tissues of the body [16]. In this study we found that cycloartenol could induce Sub-G1 cell cycle arrest and could also suppress the motility and migration of the glioma U87 cells. The p38 MAPK pathway is generally activated in cancer cells and as such is been considered an important therapeutic target for the treatment of cancers [17]. Our results indicated that cycloartenol could suppress the phosphorylation of p38, indicative of the potential of cycloartenol as anticancer agent.

Conclusion

From the findings of this study we conclude that cycloartenol inhibits the proliferation of the glioma U87 cells via induction of sub-G1 cell cycle arrest and induction p38 MAPK-mediated apoptosis. Cycloartenol may therefore prove essential in the treatment of glioma and warrants further investigation.

Acknowledgement

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Conflict of interests

The authors declare no conflict of interests.

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