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ORIGINAL ARTICLE

Anticancer effects of bishydroxycoumarin are mediated through apoptosis induction, cell migration inhibition and cell cycle arrest in human glioma cells

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Summary

Purpose: *To evaluate the cytotoxic and apoptotic effects of bishydroxycoumarin (BHC) against human glioma cells and to study their mode of action.*

Methods: *Three cells were used in the experiments. MTT and LDH assays were used to assess the cytotoxic effects of BHC while an in vitro wound healing assay was used to study the effect of BHC on cell migration. Fluorescence microscopy and annexin V-FITC assay were used to study the cellular morphology and apoptotic effects while flow cytometry in combination with propidium iodide (PI) were used to study cell cycle arrest induced by BHC.*

Results: *BHC induced substantial and dose-dependent cytotoxic effects against all three cell lines with U87MG cell line being most susceptible. BHC also inhibited cell migration and induced characteristic morphological changes including chromatin condensation, nuclear shrinkage which increased with increasing dose of BHC. The apoptotic cell population (both early and late apoptotic cells) increased with increasing dose of BHC which also induced substantial G0/G1 cell cycle growth arrest in U87MG cells.*

Conclusion: *This study provides help to elucidate the translational potential of the in vitro results to be used for further in vivo studies on human glioma using animal or human subjects. The mechanistic pathway studied in this report could be helpful in explaining the mode of action of these classes of natural products.*

Key words: *apoptosis, bishydroxycoumarin, cell cycle, cell migration, glioma*

Introduction

Malignant gliomas, together with glioblastoma multiforme (GBM), a grade IV astrocytoma, and anaplastic astrocytomas are the most common and most aggressive primary brain cancers in humans. GBM is an enormously aggressive and invasive malignancy with much quicker proliferation rate than grade III tumors [1,2]. Malignant glioma generally presents with headache, cognitive change, epilepsy, dysphasia, and progressive hemiparesis, signifying damage of normal brain cells and widespread tumor invasion. Due to the highly invasive tendency of glioma cells into surrounding non-cancerous tissues, the extensive resection is not curative. The results obtained from chemotherapy and radiotherapy regimens in malignant glioma are very disappointing due to the inherent resistance of cancer cells towards such treatments. As a result, the median survival of the malignant glioma patients is still 15 months from the time of diagnosis and most of cancer patients die within 2 years [3,4]. Currently, concomitant radiochemotherapy with temozolomide (TMZ), an alkylating agent, is used widely in China and other parts of the world, and has been shown to decrease the risk of reappearance and prolong patient survival. However, this treatment strat-

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egy is not without serious side-effects including myelosuppression, nonhematologic toxicity, and infection [5]. This problem is further enhanced by the fact that the use of temozolomide in treating malignant glioma patients is limited because of the resistance acquired by the glioma cells due to the increased activity of the DNA repair enzyme O^6 -alkylguanine-DNA alkyltransferase [6]. Therefore, there is a pressing need to search for new chemotherapeutic agents with higher activity and less likelihood of acquiring resistance against this aggressive carcinoma. A large number of plant-derived bioactive compounds have been isolated that are now widely used to treat diverse malignant tumors.

Coumarins hold a key place in the drug discovery process owing to their wide spectrum of biological activities. Coumarins are naturally occurring compounds and versatile synthetic scaffolds with potential anticancer activity also. Numerous reports have suggested the possible use of coumarins in the treatment of cancer cells. The *in vitro* study of coumarins against renal cell carcinoma indicated that coumarin and 7-hydroxycoumarin were potent cytotoxic and cytostatic agents. Furthermore, these coumarin compounds exhibited cytotoxic activity against numerous human cancer cell lines in vitro and in xenograft models [7,8].

The objective of the current study was to examine the anticancer effects of BHC-a plant-derived coumarin, against three human glioma cancer cell lines including LN18, T98G and U87MG along with evaluating its effects on apoptosis, cell migration and cell cycle arrest in U87MG cell line.

Methods

Chemicals and other reagents

BHC was purchased from Sigma Chemical Company (St. Louis. Co), and 100 mg/ml solution dissolved in DMSO was stored at -20 °C prior to use. RPMI 1640 medium, fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Hangzhou Sijiqing Biological Products Co., Ltd, China. MTT kit was obtained from Roche (USA). Annexin V-FITC-PI Apoptosis Detection Kit, and Hoechst 33258 staining kit were purchased from Beyotime Institute of Biotechnology, Shanghai, China. All other chemicals and solvents used were of the highest purity grade. Cell culture plasticware was bought from BD Falcon (USA).

Cell lines and culture conditions

Human glioma cell lines LN18, T98G and U87MG were supplied from the Shanghai Institute of Cell Resource Center of Life Science (Shanghai, China). The cells were allowed to attach for 2 hrs prior to the addition of BHC. The cells were maintained in RPMI-1640 supplemented with 10% FBS penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of CO_2 at 37°C. BHC was dissolved in DMSO at 10 mg/ml as a stock solution and diluted to the necessary concentration with fresh medium prior to use. The final DMSO concentration in the cultures was <0.1% (v/v), which did not influence cell growth when compared with that of the vehicle free controls. Cells were grown in the media containing an equivalent amount of DMSO without BHC to serve as a negative control.

MTT cell viability assay

The cell viability of the human glioma cell lines (LN18, T98G and U87MG) was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, cells were placed in 96-well culture plates (3×10^6 cells/well). After 24 hrs adherence, the cells were treated with 0, 5, 10, 25, 50, 75 and 100 µM BHC or 0.1% DMSO respectively for 24 hrs. At the end of treatment, MTT (5 mg/mL) was added to each well. The cells were incubated for another 4 hrs, and 150 µL DMSO were added to each well. Absorbance at 570 nm was measured and the growth inhibition ratio was calculated. Three independent experiments were performed. The half-maximal inhibitory concentration values (IC_{50}) were obtained from the MTT viability curves.

Lactate dehydrogenase (LDH) release assay

This assay was performed using CytoTox 96® Non Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA). The cell lines were seeded at a density of $2x10^4$ cells per well separately into 24-well plates. The cells were treated with 0, 5, 10, 25, 50, 75 and 100 µM BHC or 0.1% DMSO, respectively, for 24 hrs. After the treatment, the media from each well were collected to calculate the amount of released LDH. Following 30 min at room temperature in the dark, absorbance was recorded on a microplate reader at a wavelength of 490 nm. Spontaneous LDH release from untreated normal cells (Lc) and high level maximum release by disrupting the cells with Triton X-100 (Hc) were analyzed. The percentage of LDH release compared with the control was calculated as: [(treated mean - Lc) / (Hc - Lc)] x 100.

In vitro wound healing assay

This assay was performed using a standard method [9]. Cells ($1x10^5$ cells/ml) were seeded in a 6-well plate and incubated at 37°C until 95-100% full confluent monolayer was obtained. Subsequent to 12 hrs of starvation, a 100 ml pipette tip was used to create a straight cell free wound. Each well was washed twice with PBS to remove any debris and then exposed to

various concentrations of BHC (0, 5, 10, 25, 50, 75 and 100 μM) in a medium. After 48 hrs of incubation, the cells were fixed and stained with 5% ethanol containing 0.8% crystal violet powder for 30 min, and randomly chosen fields were photographed under a light microscope. The number of cells that migrated into the scratched area was counted.

Morphological study using fluorescence microscopy and Hoechst 33258

From the previous experiments, it was revealed that U87MG glioma cells were the most affected cells by the BHC in MTT, LDH and wound healing experiments. We selected this cell line for further in-depth studies. For studying the effects of BHC on the cellular morphology of U87MG cells, these cells were seeded into 12-well plates at a density of $1x10^5$ cells/well in 2 ml medium. After treatment with different concentrations (0, 25, 75 and 100 μM) of BHC for 48 hrs, morphological cell analysis was done by the Hoechst staining kit according to the manufacturer's instructions. After this, the cells were fixed with 3% polyoxymethylene and then incubated in Hoechst 33258 solution for 15 min in the dark. The high quality images were recorded using a UV fluorescence microscope (Olympus, Olympus Optical Co., LTD, Tokyo, Japan) using UV filter at x 200 magnification.

Quantification of apoptotic cell death by annexin V-FITC assay

Apoptosis was detected by using an annexin V-FITC apoptosis detection kit (Beyotime Institute of Biotechnology, Shanghai, China). Cells treated with or without BHC (0,25,75 and 100 μM) for 48 hrs were stained with annexin V- FITC and PI according to manufacturer's instructions. The percentage of live, apoptotic and necrotic cells was analysed by flow cytometry immediately (Becton Dickinson, San Jose, CA, USA). Data from 10^5 cells was analyzed for each sample spectrophotometrically at 405 nm with ELISA based cell death detection kits (Calbiochem, Germany).

Cell cycle analysis

U87MG cells (5×10^6) were seeded in 60-mm dishes and subjected to various concentrations (0,25,75 and 100 μM) of BHC for 48 hrs. Floating and adherent cells were collected by trypsinization and washed twice with PBS. Cells were incubated in 70% ethanol at -20°C overnight, treated with 10 μg/mL RNase A, then stained with 2.0 μg/mL of PI. Finally the stained cells were analyzed and studied by flow cytometry at 488 nm wavelength (FACS Calibur instrument; BD Biosciences, San Jose, CA, USA; equipped with Cell Quest 3.3 software).

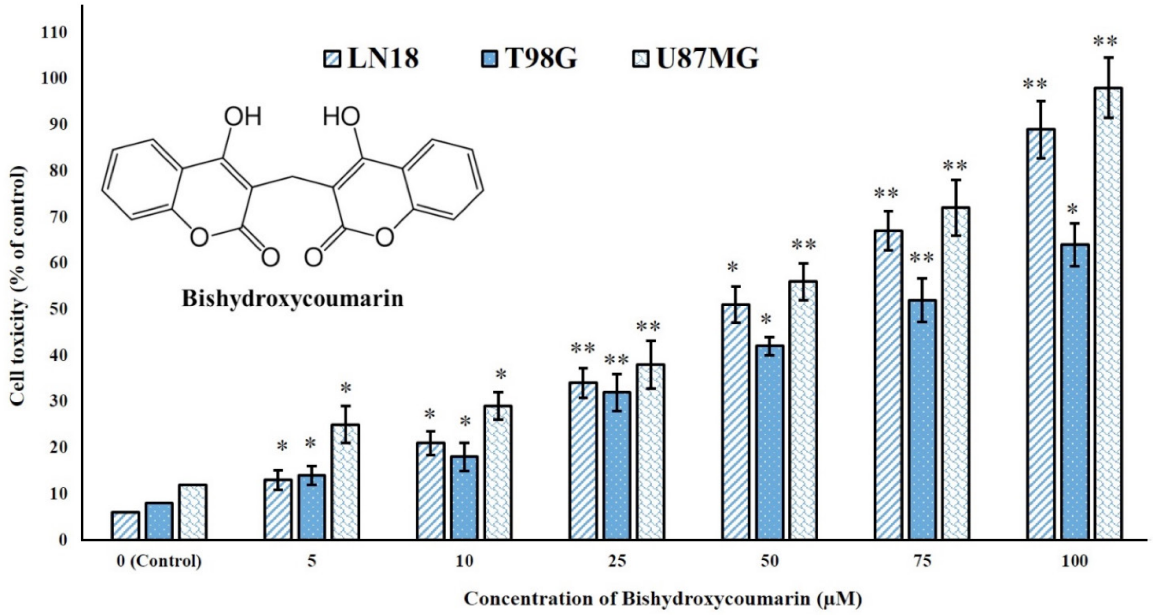

Figure 1. Effect of bishydroxycoumarin (BHC) on cytotoxicity of three human glioma cell lines LN18, T98G and U87MG. The cells were treated with BHC at the indicated concentrations for 24 hrs. Cell viability was assessed by MTT assay. The data are expressed as percentage of control and each value represents mean ± SD of three independent experiments. *p < 0.05, **p < 0.01 vs control.

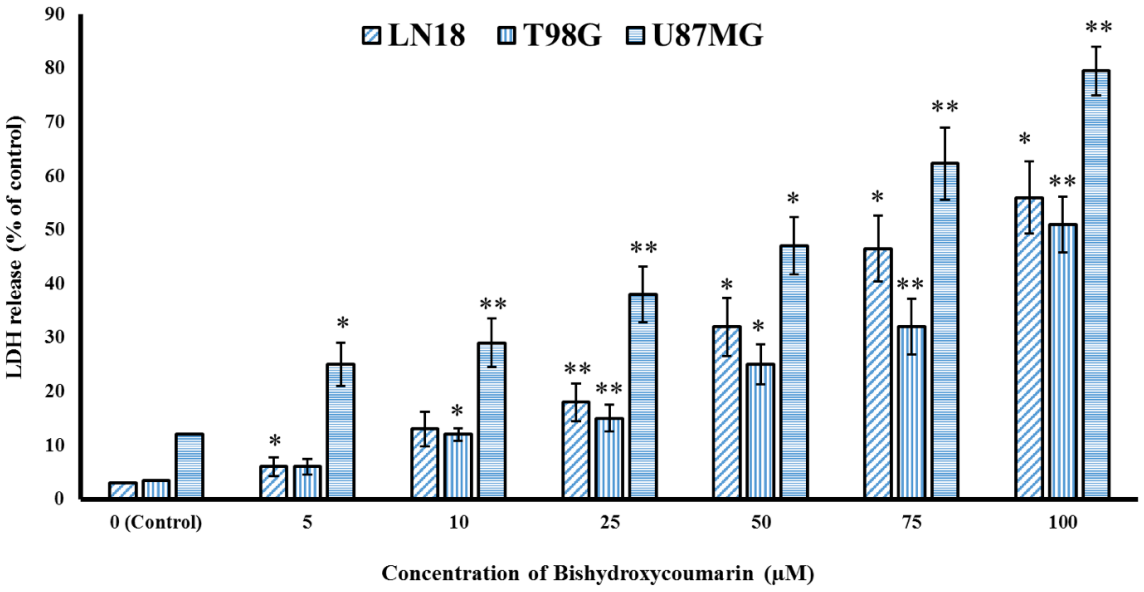

Figure 2. Cytotoxic effect of BHC. Figure shows the concentration-dependent lactate dehydrogenase (LDH) release from LN18,T98G and U87MG human glioma cells induced by 48-h bishydroxycoumarin treatment. *p<0.05, **p<0.01 vs control (0 µM).

Statistics

All data were derived from at least three independent experiments. The results were expressed as the mean ± SD. Differences between groups were analyzed using the Student's *t*-test and p<0.05 was considered statistically significant.

Results

Effects of bishydroxycoumarin on the proliferation of LN18, T98G and U87MG human glioma cells

Initially we studied the antiproliferative activity of BHC (Figure 1) on LN18, T98G and U87MG cell lines by using MTT assay using various doses (0, 5, 10, 25, 50, 75 and 100 µM) of BHC. The results revealed that BHC had potent and dose-dependent antiproliferative effects on all three cell lines. Among the three cell lines, U87MG cell line was most affected by the compound followed by LN18 cell line. T98G cell line was the least affected among the three cell lines with an IC_{50} value of 73.2 µM. LN18 cell line showed an IC_{50} of 48.5 µM while U87MG showed an IC_{50} value of 44.6 µM.

Evaluation of bishydroxycoumarin induced cell death with LDH release assay

To scrutinize whether BHC induces cell death in human glioma cells, these three cell lines were exposed to different concentrations (0, 5, 10, 25, 50, 75 and 100 µM) of BHC. The extent of cell death was measured after a 48 h incubation period using LDH release assay. Figure 2 shows the substantial BHC-induced LDH release in LN18, T98G and U87MG human glioma cells as compared to the control (*p<0.05, **p< 0.01 vs control). In this case also, a similar trend was observed with U87MG cell line being the most affected and T98G being the least affected cell line.

Effects of bishydroxycoumarin on the migration of LN18, T98G and U87MG human glioma cells

As can be seen in Figure 3, BHC reduced noticeably cell migration in all these cell lines in a concentration-dependent manner. As expected, the higher inhibition in cell migration was seen in U87MG cells followed by T98G and the least inhibition of cell migration was seen in LN18 cells.

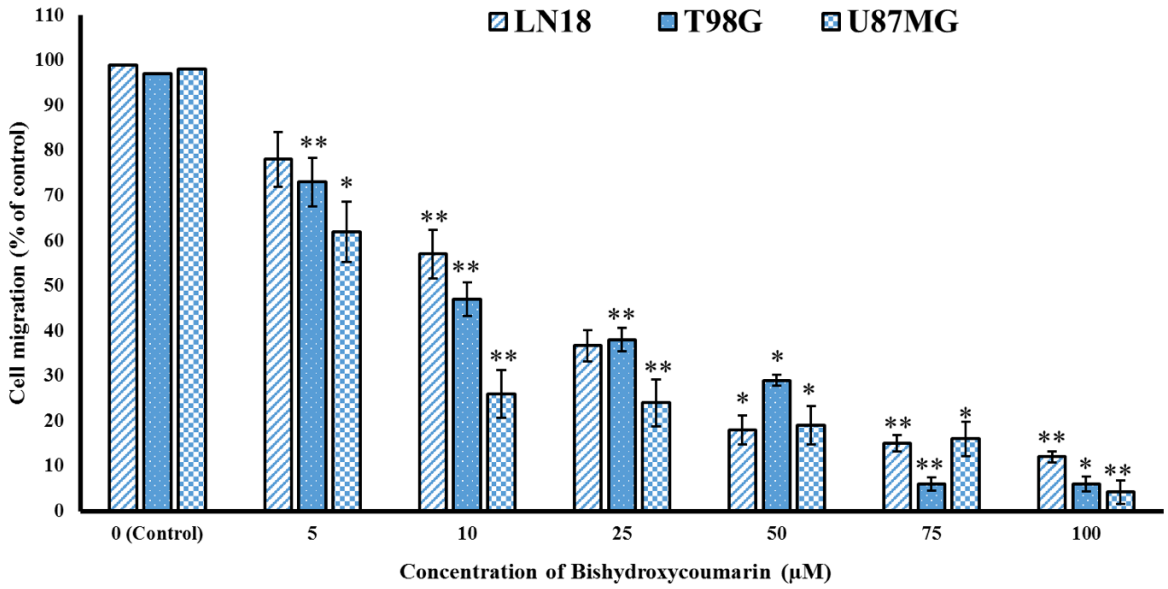

Figure 3. Graphical representation of the bishydroxycoumarin-induced reduction in the migration of LN18, T98G and U87MG human glioma cells. Data are shown as the mean ± SD of three independent experiments. *, p<0.05, **, p<0.01 vs 0 µM (control). The compound induced a dose-dependent decrease in the migration of cancer cells. U87MG cells were the most affected cells.

Morphological study using fluorescence microscopy

Following treatment with different doses of BHC for 48 hrs, the cells were investigated by fluorescence microscope. BHC-treated cells stained with Hoechst 33258 revealed chromatin condensation, fragmented nuclei and nuclear shrinkage which increased with increasing dose of the compound (Figure 4 A-D). These morphological changes are the hallmark of cellular apoptosis. The number of cells with disintegrated nuclei increase with the increased in the concentration of BHC.

Apoptosis quantification by Annexin V-FITC/PI assay

Annexin V/PI double staining was used to detect apoptosis in human glioma (U87MG) cells (Figure 5 A-D). The cells were treated with different concentration ((0, 25, 75 and 100 µM) of BHC for 48 hrs. The compound induced both early and late apoptosis in a concentration-dependent manner (Figure 5 B-D) as compared to the untreated control cells (Figure 5 A). When the cells were treated with 25, 75 and 100 µM for 48 hrs, the average proportion of Annexin V-staining positive cells (total apoptotic cells) significantly increased from 8.11% in control to 23.47%, 45.4% and 73.4% respectively.

Effect of bishydroxycoumarin on cell cycle progression

Apoptosis and cell cycle are closely related biochemical processes, and any disruption in cell cycle progression may finally lead to apoptotic cell death. In order to have a mechanistic overview of the growth inhibitory effect exerted by BHC in U87MG cancer cells, flow cytometry analysis was carried out to detect whether this compound induces cell cycle arrest in this cell line. The results showed that treatment with different concentrations of the compound for 48 hrs induced significant G0/G1-phase arrest in U87MG cells. As it is obvious in Figure 6, following different doses (0, 25, 75 and 100 µM) of the compound to the glioma cells, considerable G0/G1 cell cycle arrest was seen. At low doses of the compound, apoptosis was not significant, however at 75 and 100 µM concentrations, the fraction of cells undergoing apoptosis (G0/G1) increased considerably.

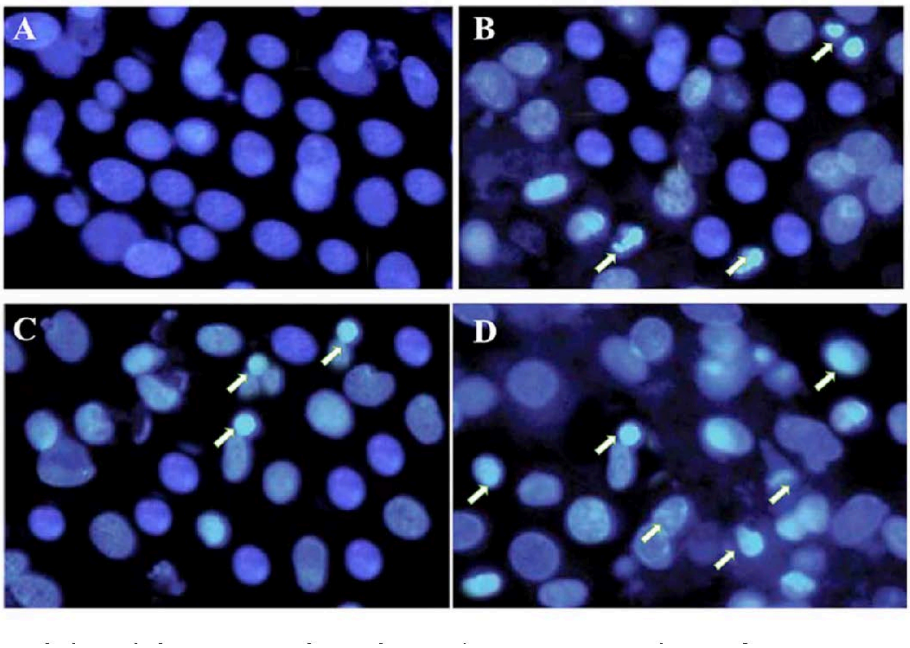

Figure 4. Morphological observation with Hoechst 33258 staining at actual magnification x200. U87MG cells were treated without **(A)** and with bishydroxycoumarin 25 μM **(B)**, 75 μM **(C)**, and 100 μM **(D)** for 48 hrs. Nuclear morphological changes can be clearly observed at higher concentrations (magnification x200; arrows indicate chromatin condensation).

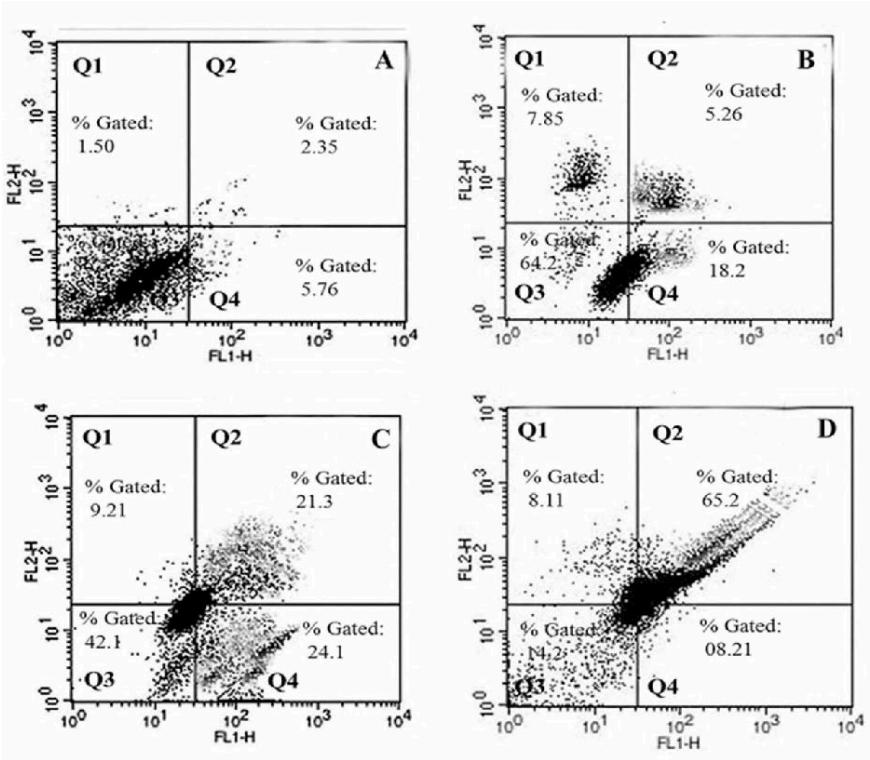

Figure 5. Bishydroxycoumarin induces apoptosis in U87MG cancer cells. Apoptotic cells were evaluated by flow cytometry using Annexin V and propidium iodide (PI) staining. Cells in the lower left quadrant (Q3) (Annexin V-FITC-/PI-) are viable, those in the lower right quadrant (Q4) are early apoptotic and those in the upper right quadrant (Q2) are late apoptotic, while those in the upper left quadrant (Q1) are necrotic. The percentages of each of these cells at 0 μM **(A)**, 25μM **(B)**, 75 μM **(C)** and 100 μM **(D)** concentration of bishydroxycoumarin are given

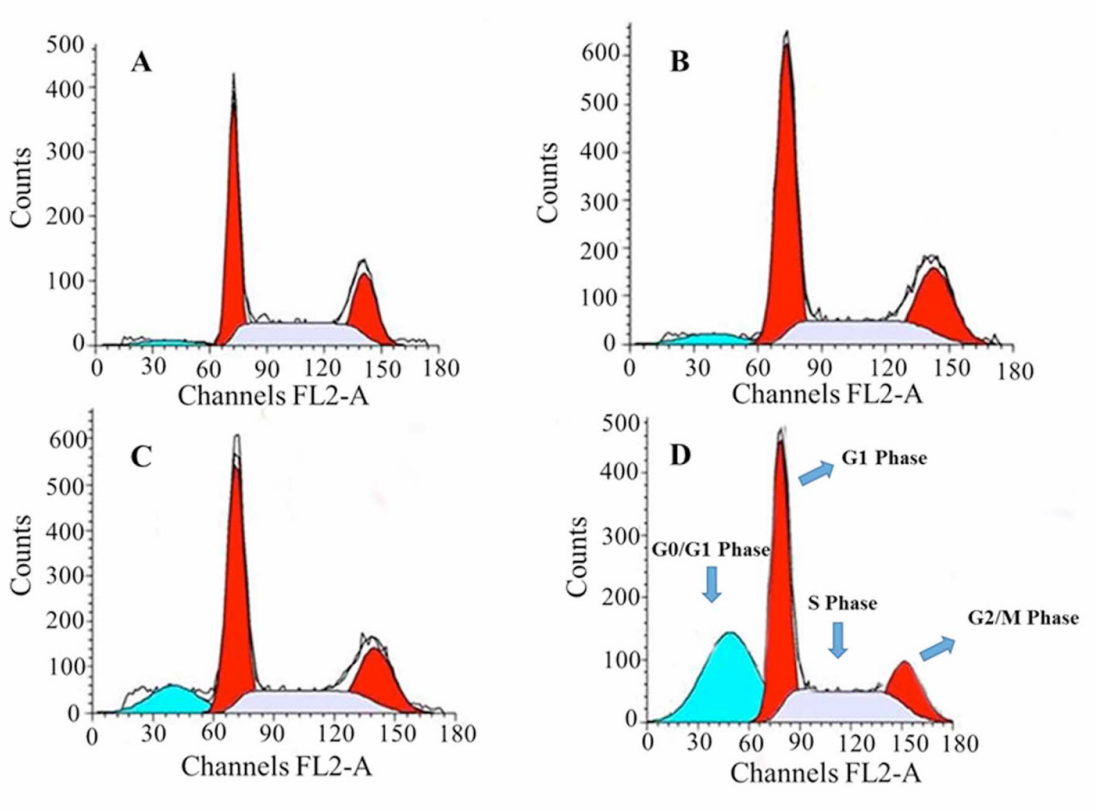

Figure 6. Effect of different concentrations of bishydroxycoumarin on the cell cycle phase distribution of human glioma U87MG cells using flow cytometry. **A**, **B**, **C** and **D**, show the effect of 0, 25, 75 and 100 μM dose of the compound on different cell cycle phases, respectively. Bishydroxycoumarin induced dose-dependent increase in the G0/G1 cell population (apoptotic cells).

Discussion

Coumarins belong to benzopyrone family of natural products consisting of a benzene ring which is attached to a pyrone ring. Coumarins are biologically active compounds exhibiting a wide range of biological activities including antibacterial, antimutagenic [10], antithrombotic [11] and anticancer activities among many others. Coumarins and their derivatives have been reported to possess anticancer activities against a range of cancers including leukaemia, renal cell carcinoma, breast cancer, malignant melanoma, prostate cancer etc [12-16]. Coumarins, when applied in combination with radiotherapy and surgery as a chemotherapeutic agent, provide best results. They not only treat tumor but also reduce the side effects of radiotherapy [17].

BHC(3,3'-methylenebis(4-hydroxy-2H-chromen-2-one) also known as dicoumarol is a naturally occurring, bitter-tasting coumarin derivative of both plant and fungal origin. It is metabolized from coumarin to the sweet clover (*Melilotus alba* and *Melilotus officinalis*) by molds, such as *Penicillium nigricans* and *Penicillium jensi*. Dicoumarol and its synthetic derivative (coumadin) have been shown to inhibit cancer metastases in *in vivo* experimental models [18]. The coumarin compounds like dicoumarol have not received much attention for cancer treatment possibly due to less information available on the cellular mechanism of action of these compounds.

Keeping this in mind, the objective of the present study was to evaluate the antitumor activity of BHC against three human glioma cancer cell lines including LN18, T98G and U87MG. We also evaluated its mode of action by studying the effect of BHC on cell migration, cell cycle phase distribution, cellular morphology as well as apoptosis in U87MG cells. BHC was able to induce cytotoxic effects in all the three cell lines as revealed by MTT and LDH assays. U87MG cell line was the most affected cell line followed by LN18,

while as T98G cell line was less susceptible to the BHC treatment. In case of *in vitro* wound healing assay, it was observed that BHC also inhibited cell migration of all three human glioma cell lines with U87MG cell line being the most susceptible. From these experiments, we selected U87MG cell line for further in depth studies based on its significant toxicity induced by BHC along with cell migration inhibition results. Fluorescence microscopy using Hoechst 33258 revealed that BHC induced chromatin condensation and significant nuclear morphological changes characteristic of apoptosis. Furthermore, we used annexin V/PI staining to detect early and late apoptotic cells generated by the treatment of BHC. The cells were treated with different concentration (0, 25, 75 and 100 μM) of BHC for 48 hrs. The compound induced both early and late apoptosis in a concentration-dependent manner. The percentage of apoptotic cells increased from 8.11% in the control to 23.47%, 45.4% and 73.4% respectively. We also evaluated the effect of BHC on cell cycle arrest in U87MG glioma cells using flow cytometry and PI. Treatment with different concentrations of the compound for 48 hrs induced significant G0/G1-phase arrest in U87MG cells. The rise in the sub-G1 cell population (hypodiploid DNA content) may be due to DNA fragmentation which eventually results in apoptotic cell death.

Conclusion

To the best of our knowledge, the current study involving BHC constitutes the first report on human glioma cells. The results of the study provide the translational potential of our in vitro results to be used for further *in vivo* studies using animals or human subjects. The mechanistic pathway studied in this report could be helpful in explaining the mode of action of these classes of natural products. In addition, the current treatment regimens used in human glioma present serious side effects coupled with the growing menace of drug resistance. BHC could be further developed as a possible anticancer agent or used in combination with other commonly used anticancer drugs for glioma like temozolomide for enhancing its efficacy.

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