

## RESEARCH ARTICLE

# Inhibitory Effect of Benzyl Isothiocyanate on Proliferation *in vitro* of Human Glioma Cells

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## Abstract

Malignant glioma, also known as brain cancer, is the most common intracranial tumor, having an extremely high mortality and recurrence rate. The survival rate of the affected patients is very low and treatment is difficult. Hence, growth inhibition of glioma has become a hot topic in the study of brain cancer treatment. Among the various isothiocyanate compounds, it has been confirmed that benzyl isothiocyanate (BITC) can inhibit the growth of a variety of tumors, including leukemia, glioma and lung cancer, both inside and outside the body. This study explored inhibitory effects of BITC on human glioma U87MG cells, as well as potential mechanisms. It was found that BITC could inhibit proliferation, induce apoptosis and arrest cell cycling of U87MG cells. In addition, it inhibited the expression of SOD and GSH, and caused oxidative stress to tumor cells. Therefore, it is believed that BITC can inhibit the growth of U87MG cells outside the body. Its mechanism may be related to the fact that BITC can cause oxidative stress to tumor cells.

**Keywords:** Glioma - benzyl isothiocyanate - oxidative stress - preventive potential

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## Introduction

The treatment of malignant glioma is mainly relying on surgical excision at present, supported by postoperative radiotherapy and chemotherapy. Therefore, it is necessary to develop new medicine in order to improve the treatment efficacy. Recent studies have found that a large number of edible cruciferous plants, such as broccoli, cabbage and water celery, can reduce the risk of cancer. It has also been confirmed that the isothiocyanates compounds contained in these plants have anti-tumor ability. Up to now, researchers have studied more than 20 kinds of natural or synthetic isothiocyanate compounds and observed that they have a great inhibition effect on a variety of tumors. Recent epidemiological and experimental studies have also shown that isothiocyanate compounds possess the potential to be developed into chemotherapy medicine, but the exact mechanism of their anti-tumor activity has not yet been fully understood (Kim et al., 2012; Pawlik et al., 2012; Zandalinas et al., 2012). Therefore, this study intended to explore the inhibition effect of benzyl isothiocyanate (BITC) on human glioma U87MG cells, as well as its potential mechanism. It will provide a certain level of theoretical and scientific basis for future research.

## Materials and Methods

### Cell culture

Culture the human glioma U87MG cell lines in the DMEM complete medium (containing 10% FCS, 100

µg/mL streptomycin and 100 µg/mL penicillin) under the conditions of 37 °C, 5% CO<sub>2</sub>. Take cells in the experimental logarithmic phase to perform test.

### Effects of BITC on proliferation of human glioma U87MG cells

Culture the human glioma U87MG cells on a 96-hole plate (5×10<sup>3</sup> cells / hole) under the conditions of 37°C, 5% CO<sub>2</sub> for 24h. After the cells are attached, add BITC medium with the final concentration of 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 µM respectively into the cell samples; add the medium with an equal volume of PBS into the negative control group (six multiple-holes for each dose). Then, culture the cells in the incubator again for a continuous 72 h. After removing the supernatant carefully, add 200 µL plasma-free medium and 40 µL MTS respectively into the cell samples. Use aluminum foil to wrap the plate and culture for another 4h. Thereafter, extract the supernatant and use the enzyme-labeled meter to measure its OD value at 490 nm; calculate the medicine IC<sub>50</sub>. Repeat the experiment for three times and take the mean value. The tumor growth inhibition rate (%) = (1 - the OD value of the medicine-treated group / the OD value of the negative control group) × 100%.

### Effects of BITC on apoptosis of human glioma U87MG cells

Culture the human glioma U87MG cells on a 6-hole plate (1.5×10<sup>5</sup> cells / hole) under the conditions of 37 °C, 5% CO<sub>2</sub> for 24 h. Then, add 10 µM and 20 µM BITC into

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the cell samples respectively; add PBS into the negative control group. 24h later, after being digested by trypsin, take 100µL cell suspension, Annexin-V-FITC and 10 µL PL and incubate the samples in dark environment for 10 min. Use the flow cytometer to perform test.

*Effects of BITC on cell cycling of human glioma U87MG cells*

Culture the human glioma U87MG cells on a 6-hole plate (1.5×10<sup>5</sup> cells / hole) under the conditions of 37 °C, 5% CO<sub>2</sub> for 24 h. Then, add 2 µM and 5 µM BITC into the cell samples respectively; add PBS into the negative control group. 24h later, after being digested by trypsin, use 70% cold ethanol to fix the samples at 4 °C; rest for overnight. Perform centrifugation to remove ethanol completely. Add Rnase A into the samples and incubate at 37 °C in dark environment for 15 min. Add 50 µg/mL PI and 500 µl dye solution into the samples. Incubate at 37 °C in dark environment for 30 min. Then, use the flow cytometer to perform test.

*Effects of BITC on ROS expression of human glioma U87MG cells*

Culture the human glioma U87MG cells on a 6-hole plate (1.5×10<sup>5</sup> cells / hole) under the conditions of 37 °C, 5% CO<sub>2</sub> for 24 h. Then, add 2 µM and 5 µM BITC into the cell samples respectively; add PBS into the negative control group. 24 h later, after being digested by trypsin, take 100 µl cell suspension and add in 5 µL intracellular reactive oxygen detection probe DCFH-DA. Incubate the samples at room temperature in dark environment for 30 min. Then, use the flow cytometer to perform test.

*Effects of BITC on GSH expression of human glioma U87MG cells*

Culture the human glioma U87MG cells on a 6-hole plate (1.5×10<sup>5</sup> cells / hole) under the conditions of 37 °C, 5% CO<sub>2</sub> for 24 h. Then, add 2 µM and 5 µM BITC into the cell samples respectively; add PBS into the negative control group. 24 h later, after being digested by trypsin, the cells shall be lysed. Then, use the enzyme-labeled meter to detect the intracellular GSH expression in accordance with the operation instructions.

*Effects of BITC on SOD activity of human glioma U87MG cells*

Culture the human glioma U87MG cells on a 6-hole plate (1.5×10<sup>5</sup> cells / hole) under the conditions of 37 °C, 5% CO<sub>2</sub> for 24 h. Then, add 2 µM and 5 µM BITC into the cell samples respectively; add PBS into the negative control group. 24h later, replace the medium with the fresh plasma-free medium and culture for 24h. Extract the supernatant and use the enzyme-labeled meter to detect the intracellular SOD activity in accordance with the operation instructions.

*Effects of BITC on caspase-3 activity of human glioma U87MG cells*

Culture the human glioma U87MG cells on a 6-hole plate (1.5×10<sup>5</sup> cells/hole) under the conditions of 37 °C, 5% CO<sub>2</sub> for 24 h. Then, add 2 µM and 5 µM BITC into

the cell samples respectively; add PBS into the negative control group. 24 h later, after being digested by trypsin, the cells shall be lysed. Perform centrifugation at 4 °C and 10,000g/10min; extract the supernatant to obtain the total protein of tissues. Perform electrophoresis in 12% SDS polyacrylamide gel; then, transfer the protein samples to a PVDF membrane. Use 4 °C, 5% skimmed milk to seal the samples and rest for overnight. Add first antibody (1:500) and β-actin (1:5000) into the samples, respectively. Then, incubate the samples at 4 °C for overnight. Use IgG (1:2000) that is labeled with horseradish peroxidase to incubate the samples at room temperature for 1h. Develop and show image.

*Statistical methods*

The experimental data is analyzed by SPSS11.5 statistical software. All data is expressed in the form of mean ± standard deviation. One-way ANOVA is adopted to compare the data, with considering P < 0.05 as the standard of statistically significant. Each experiment is repeated for three times.

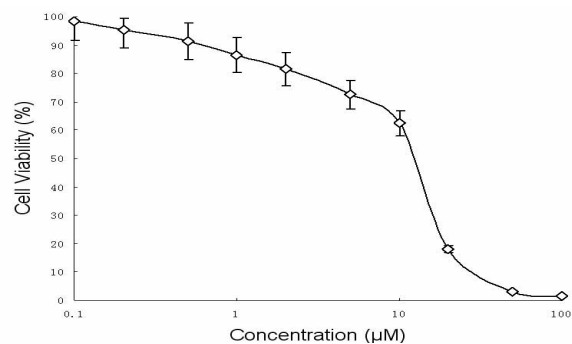
**Results**

*Effect of BITC on proliferation of human glioma U87MG cells*

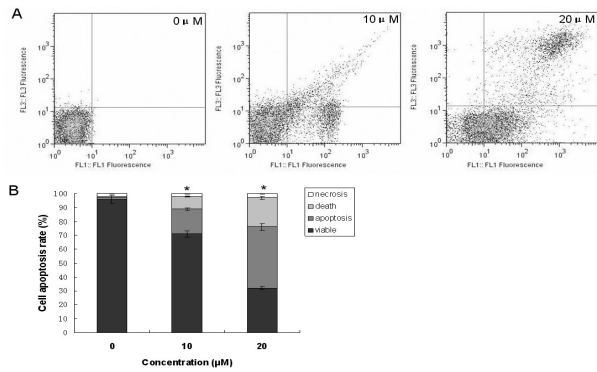
The MTS method was applied to observe the effect of BITC on the in-vitro growth of human glioma U87MG cells. The experimental results showed that: after BITC taking effect on human glioma U87MG cells for 72 h, cell proliferation was inhibited and exhibited the dose-response relationship; its IC<sub>50</sub> was equal to 15.2 µM (Figure 1). After the cell lines being treated by 2 µmol/L BITC for 24 h, the inhibition rate was less than 5%; after being treated by 5 µmol/L BITC for 24 h, the inhibition rate was less than 15%. The U87MG cells were in the non-toxic and low-toxic concentration, respectively. Therefore, BITC with the concentration of 2 µmol/L and 5 µmol/L were used to test the cell cycle, the ROS, SOD and GSH expression/activity and the caspase-3 activity, in order to eliminate the interference of cell growth inhibition and apoptosis on the above mentioned experiments.

*Effects of BITC on apoptosis of human glioma U87MG cells*

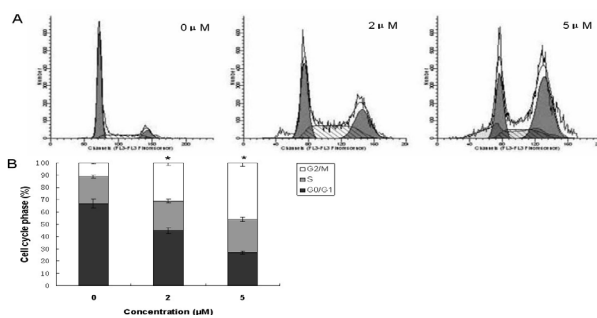
The Annexin-V-FITC and PI double staining test showed that after treating the cells with BITC for 24 h,



**Figure 1. The Inhibitory Effect of BITC on the U87MG Cell Line Proliferation.** The data was presented as mean ± SD, bars indicate SD, n= 6



**Figure 2. The Apoptosis-induced Effect of BITC on the U87MG Cell Line.** A: The flow cytometry results in apoptosis effect-induced of U87MG cells; B: The apoptosis effect of BITC on the U87MG cell line. Bars indicate SD. n=3. \* $P < 0.05$  compared with control group



**Figure 3. The Effect of BITC on the Cell Cycle of U87MG Cell Line.** A: The flow cytometry results in cell cycle effect of U87MG cells; B: The cell cycle effect of BITC on the U87MG cell line. Bars indicate SD. n=3. \* $P < 0.05$  compared with control group

the apoptosis rate caused by 10  $\mu\text{M}$  and 20  $\mu\text{M}$  BITC on the U87MG cells were 29.3% and 68.6% respectively, which was significantly higher than 3.2% of the control group ( $P < 0.05$ ) (Figure 2).

#### Effects of BITC on cell cycling of human glioma U87MG cells

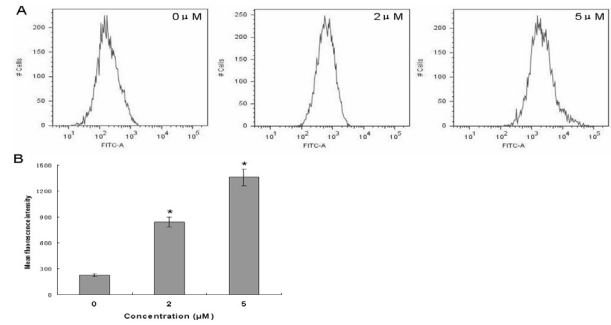
The measurement result of the flow cytometer showed that after treating the cells with 2  $\mu\text{M}$  and 5  $\mu\text{M}$  BITC for 24 h, the cell cycle distribution of the tumor was changed ( $P < 0.05$ ). Specifically, the proportion of phase G2/M was increased, while the proportion of phase G0/G1 was decreased ( $P < 0.05$ ) (Figure 3), suggesting that BITC can arrest the cell cycle of U87MG cells at phase G2/M.

#### Effects of BITC on ROS expression of human glioma U87MG cells

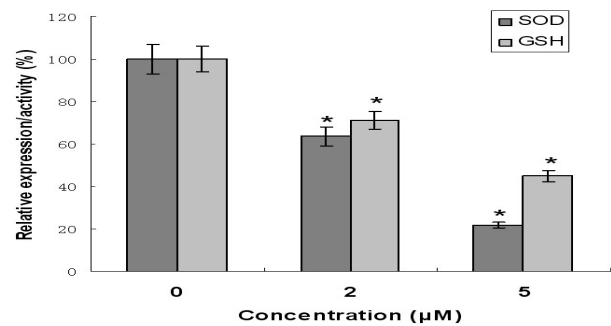
The result of the flow cytometer showed that after treating the U87MG cells with 2  $\mu\text{M}$  and 5  $\mu\text{M}$  BITC for 24 h, the ROS expression of the tumor cells was significantly increased; specifically, the ROS expression of the 2  $\mu\text{M}$  group and 5  $\mu\text{M}$  group was 3.76-fold and 6.07-fold of that of the control group, respectively ( $P < 0.05$ ) (Figure 4).

#### Effects of BITC on GSH expression and SOD activity of human glioma U87MG cells

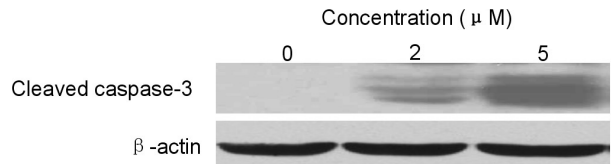
The result showed that after treating the U87MG cells with 2  $\mu\text{M}$  and 5  $\mu\text{M}$  BITC for 24 h, the GSH expression



**Figure 4. The Effect of BITC on the ROS Expression of U87MG Cell Line.** A: The flow cytometry results in ROS expression of U87MG cells; B: The ROS expression of BITC on the U87MG cell line. Bars indicate SD. n=3. \* $P < 0.05$  compared with control group



**Figure 5. The Effect of BITC on GSH Expression and SOD Activity of U87MG Cell Line.** A: The effect of BITC on expression of GSH in the U87MG cell line. B: The effect of BITC on activity of SOD in the U87MG cell line. Bars indicate SD. n=6. \* $P < 0.05$  compared with control group



**Figure 6. The Effect of BITC on Cleaved Caspase-3 Expression of U87MG Cell Line.** Western blot showed increased cleaved caspase-3 expression in U87MG cell line with different concentration BITC treatment.  $\beta$ -actin was used as an internal control for loading

and SOD activity expression of the tumor cells were significantly decreased. The GSH expression of the 2  $\mu\text{M}$  group and 5  $\mu\text{M}$  group was 71.3% and 44.9% of that of the control group, respectively ( $P < 0.05$ ); the SOD activity of the 2  $\mu\text{M}$  group and 5  $\mu\text{M}$  group was 63.5% and 21.8% of that of the control group, respectively ( $P < 0.05$ ) (Figure 5).

#### Effects of BITC on caspase-3 activity of human glioma U87MG cells

The result showed that after treating the U87MG cells with 2  $\mu\text{M}$  and 5  $\mu\text{M}$  BITC for 24 h, the caspase-3 activity of the tumor cells was significantly decreased ( $P < 0.05$ ) (Figure 6).

## Discussion

Malignant glioma, also known as brain cancer, is the most common intracranial tumor, having an extremely high mortality. In recent years, although the various

treatment approaches for malignant glioma, including surgical operation, radiotherapy, chemotherapy and targeted therapy, have been undergoing a rapid development, there are still a series of problems, such as high recurrence rate, low survival rate and high treatment difficulty, to be further addressed. The survival cycle of the patients is merely about 14 months, and only 5% of the patients can survive for more than 5 years (Tabatabai et al., 2012). The high recurrence rate and low survival rate of malignant glioma is closely related to the invasive growth of the tumor and the special structure of the brain. Glioma is often occurred under the cortex, featured with infiltrative growth. It may transfer to a wide range of locations and often invades several brain lobes, as well as the deep structure. Sometimes, it can even injure the contralateral hemisphere via callosum and grow together with healthy brain tissues, so that the tumor is difficult to be completely removed by surgery. Meanwhile, for some patients, the tumor locations are inaccessible or serious consequences may occur during the postoperative phase, making the surgery operation impossible (Fidler, 2011). Therefore, chemotherapy of glioma has drawn increasingly more attention nowadays. This study observed that BITC has an inhibition effect on the proliferation of U87MG cells; it can also arrest the cell cycle and induce the apoptosis of the human glioma U87MG cells, indicating that BITC has the anti-tumor effect.

Apoptosis is a kind of biological phenomenon of programmed cell death. Anti-tumor medicine plays the anti-tumor effect by inducing the apoptosis of tumor cells. Cell cycle arrest and oxidative stress are the main causes of apoptosis. The nature of cell proliferation is to realize DNA replication constantly through cell cycle; when cell cycle arrest occurs, the proliferation of tumor cells will be inhibited (Deeb et al., 2012). This study showed that BITC can arrest the cell cycle at phase G2/M, so that cells cannot enter phase G0/G1 and phase S to conduct DNA synthesis and repair. As a result, the tumor cells cannot enter the next cycle, and thereby, the proliferation of tumor cells is inhibited and the cell apoptotic program is initiated.

Superoxide Dismutase (SOD) is an important oxygen free radical scavenger in the enzymatic defense system, which is able to catalyze the transformation of superoxide into oxygen and enzyme of hydrogen peroxide via disproportionation reaction. Glutathione (GSH), which is formed by the integration of glutamic acid, cysteine and glycine, is a kind of tripeptide containing mercapto. It possesses the functions of anti-oxidation and detoxification. Both of these two elements play an important role in the biochemical defense system of human body, able to remove free radical in the body. As an important anti-oxidant, they can protect the mercapto in the molecules of many proteins and enzymes and eliminate the harmful substances produced by the organisms in the metabolism process. Meanwhile, they can also resist or block the cell injury caused by oxygen free radicals and repair the injured cells. However, SOD and GSH may limit the effect of the anti-tumor medicine (Deng et al., 2012; Kohsaka et al., 2013). This study showed that BITC can reduce SOD activity and GSH expression, which is conducive for strengthening the anti-tumor activity.

Oxidative stress is another cause of tumor cell apoptosis (Gayathri R et al., 2009). Oxidative stress can activate the cascade reaction of the cysteine aspartate acid specific protein kinase and the caspase-3 activity of the cells, which may lead to DNA breaks. As the tumor cell cycle is arrested at phase G2/M, both the synthesis and repair capacity of DNA is relatively low, and thus, apoptosis occurs in tumor cells (Martinez-Outschoorn et al., 2012; Noreen et al., 2013). In this study, it was observed that BITC may increase ROS expression in the tumor cells, suggesting that BITC can activate caspase-3 activity by affecting the cell cycle and inhibiting the SOD activity and the GSH expression, so as to accelerate the apoptosis of human glioma U87MG cells. Its mechanism may be related to the fact that BITC can cause oxidative stress to tumor cells.

In summary, BITC, which can induce apoptosis through oxidative stress, is expected to become a new anti-tumor medicine. However, further studies are needed to investigate the mechanism of BITC for arresting cell cycle and its impact on intracellular signal transduction, in order to provide an experimental basis for clinical application.

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