

Dihydroartemisinin increases temozolomide efficacy in glioma cells by inducing autophagy

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Abstract. Artemisinin, a powerful antimalarial medicine, is extracted from the Chinese herb, *Artemisia annua* L., and has the ability to inhibit the proliferation of cancer cells. Dihydroartemisinin (DHA), the major active metabolite of artemisinin, is able to inhibit the growth of a variety of types of human cancer. However, the effect of DHA on human glioma cells remains unclear. The aim of the present study was to investigate the effect of DHA on the proliferation of glioma cells, and whether DHA was able to enhance temozolomide (TMZ) sensitivity *in vitro* and *in vivo*. In total, 10 human glioma cell lines were used to analyze the growth inhibition ability of DHA by MTT assay. The typical autophagic vacuoles were monitored by the application of the autofluorescent agent, monodansylcadaverine. Western blotting was used to detect markers of apoptosis and autophagy, namely Caspase-3, Beclin-1 and LC3-B. The combination efficiency of DHA and TMZ was assessed *in vitro* and *in vivo*. The half maximal inhibitory concentration (IC₅₀) of DHA differed among the ten human glioma cell lines. The number of autophagic vacuoles was higher in DHA-treated SKMG-4 cells; this was highest of all cell lines analyzed. The expression of autophagy molecular markers, Beclin-1 and LC3-B, was increased following DHA treatment, while no significant alteration was detected in the expression of apoptotic marker Caspase-3. When combined with DHA, the IC₅₀ of TMZ decreased significantly in the four glioma cell lines analyzed. Furthermore, DHA enhanced the tumor inhibition ability of

TMZ in tumor-burdened mice. The results of the present study demonstrated that DHA inhibited the proliferation of glioma cells and enhanced the tumor inhibition efficacy of TMZ *in vitro* and *in vivo* through the induction of autophagy.

Introduction

Glioma is the most common type of primary brain tumor, accounting for ~80% of all brain and central nervous system tumors. Glioma is divided into four grades (grade I-IV) according to criterion established by the World Health Organization. The survival rate of low-grade glioma (grade II) is higher than that of high-grade glioma (anaplastic astrocytomas and glioblastoma multiforme), at ~47% (1). High-grade gliomas are the most malignant tumors and are characterized by high cellularity, mitotic activity, vascular proliferation, central necrosis and aggressive invasion into normal brain tissue (2). Despite the combination of maximal tumor resection, concurrent radiotherapy, adjuvant chemotherapy and molecular targeted therapy for the treatment of high grade glioma, the life expectancy of patients remains poor, at just 12-14 months (3-6). Therefore, the identification of novel, effective therapies for the treatment of gliomas is required.

Artemisinin is derived from a traditional Chinese herb, which is used for the treatment of fever and malaria (7). Previous studies have revealed that artemisinin is able to inhibit the progression of chronic myelogenous leukemia, pancreatic adenocarcinoma, colon cancer, prostate cancer, non-small cell lung cancer and breast malignancies at low concentrations (8-10). Artemisinin and its derivatives have also been used in the screening of anticancer drugs by the National Cancer Institute (NCI) (11).

Dihydroartemisinin (DHA), the major active metabolite of artemisinin, has been found to inhibit the growth of cancer cells in a variety of cancers (12-15). Xie *et al* (16) reported that the concentration of DHA was two-fold higher in the brain than in the plasma, which indicates that DHA is able to penetrate the brain-blood barrier. DHA triggers the production of reactive oxygen species (ROS) and inhibits the activity of glutathione-S-transferase, thereby promoting the radiosensitivity of glioma cells (17). Furthermore, DHA has been found

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to potentiate the cytotoxic effect of temozolomide (TMZ) in rat C6 glioma cells (18). However, whether DHA is able to enhance the efficiency of TMZ in human glioma cells has remained to be elucidated. The present study aimed to assess the effect of DHA on glioma cell lines as well as whether DHA could enhance the cell response to TMZ.

Materials and methods

Cell lines and reagents. In total, 10 human glioma cell lines, namely MGR1, MGR3, SF295, SKMG-1, SKMG-4, T98G, U251, U251/CP2, U373 and U87, were used in the present study. All cells were maintained in Dulbecco's modified Eagle's medium /F-12 (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Chalfont, UK) and 1.0% penicillin/streptomycin (Gibco Life Technologies) in a humidified incubator at 37°C with 5% CO₂. DHA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; 20 µg/ml) and stored at -20°C in the dark. TMZ (Sigma-Aldrich) was dissolved in DMSO, (stock concentration, 0.2 M) and stored at -20°C.

Cytotoxicity assay. The glioma cells were seeded into 96-well plates at a density of 5x10³ cells/well and incubated overnight at 37°C. Next, the cells were treated with DHA at serial concentrations (0, 0.3125, 0.625, 1.25, 2.5, 5.0, 10.0 and 20.0 µg/ml) for 72 h. Cell viability was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In order to detect the effect of DHA on TMZ efficiency, the cells were treated with 2 µM DHA in combination with various concentrations of TMZ (ranging between 31.25 and 2,000 µM). Following 72 h of incubation, the MTT assay was performed and the half maximal inhibitory concentration (IC₅₀) was calculated. Briefly, MTT (Sigma-Aldrich) was added to cells following treatment and incubated for 4 h at 37°C. DMSO was subsequently added and absorbance was measured by microplate reader (Multiskan MK3; Thermo Fisher Scientific, Waltham, MA, USA) after shaking for ~30 sec.

Western blot analysis. The cell lysates were collected using radioimmunoprecipitation assay lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl and 10 mM Na₂HPO₄; pH 7.2; Beyotime Institute of Biotechnology, Japan), supplemented with a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Immunoblotting was performed as previously described (19). Rabbit anti-human antibodies against LC3-B (polyclonal; catalog no. ab48394; dilution, 1:1,000; Abcam, Cambridge, MA, USA), Beclin-1 (monoclonal; catalog no. ab51031; dilution, 1:750; Abcam) and Caspase-3 (polyclonal; catalog no. ab90437; dilution, 1:100; Abcam) were added and incubated overnight at 4°C. A mouse anti-human β-actin antibody (catalog no. AA128; dilution, 1:1,000; Beyotime Institute of Biotechnology) was loaded as the internal control. The membranes were then washed three times with phosphate-buffered saline (PBS). Horseradish peroxidase-conjugated goat anti-mouse (dilution, 1:1,000; catalog no. A0216; Beyotime Institute of Biotechnology) and goat anti-rabbit (dilution, 1:1,000; catalog no. 7074; Cell Signaling

Technology Inc., Danvers, MA, USA) secondary antibodies were applied for 30 min at room temperature. The membranes were then washed three times with PBS and incubated with enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Next, the membranes were exposed to ECL sensitive films (Hyperfilm ECL; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), and developed using an OPTIMAX X-Ray film processor (Protec GmbH & Co. KG, Oberstenfeld, Germany). All protein bands were normalized to β-actin, which was used as the internal loading control.

Monodansylcadaverine (MDC) fluorescence staining. The glioma SKMG-4 cells, which are sensitive to DHA, were seeded into a 30-mm culture dish and treated with 2 µg/ml DHA for 24 h. MDC (Sigma-Aldrich), at a concentration of 0.05 mM, was then added for 10 min. Next, the cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 min and washed twice with phosphate-buffered saline. The cytoplasmic autophagic vacuoles were then observed under a fluorescence microscope (Olympus BX61; Olympus Corp., Tokyo, Japan).

In vivo glioma formation assay. The U251 cells were maintained in standard culture medium, adjusted to 5x10⁶ cells/ml. Next, 300 µl cell suspension was injected into the axillary region of the nude mice (age, 4-5 weeks; mean weight, 22.6 g). Mice were maintained in specific-pathogen-free grade room, with 6 mice per cage, at 18-29°C and with light from 7:30 am to 19:30 pm. Two weeks later, the tumor masses were resected, cut into sections measuring 2 mm³ and transplanted subcutaneously into the backs of a second set of experimental nude mice. These mice were randomly separated into four groups (n=10) and treated for 5 continuous days with either DMSO (control), 50 mg/kg DHA only, 50 mg/kg TMZ only, or 50 mg/kg DHA plus 50 mg/kg TMZ. The tumor sizes were measured using a vernier caliper every three days during the following month. The tumor volumes (TV) were calculated according to the following formula: TV = 1/2 x length x width² (20). The present study was approved by the ethics committee of Sun Yat-sen University Cancer Center (Guangzhou, China) according to Institutional Animal Care and Use Committee protocols. Chloral hydrate was applied for anesthesia (10%; 80 µl per mouse), and mice were sacrificed by cervical dislocation.

Statistical analysis. The data is expressed as the mean ± standard error of the mean. Student's t-test was used to determine any statistically significant differences. P-values are two sided, with P<0.05 considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA).

Results

DHA is cytotoxic to glioma cells in vitro. The IC₅₀ of ten glioma cell lines treated with serial concentrations DHA was calculated. Following treatment, the SKMG-4 cells appeared to be low in density and exhibit abnormal morphologies compared with the non-treated cells (Fig. 1A and B). The extent of DHA toxicity was mild and differed amongst the glioma cell lines (Fig. 1C). The IC₅₀ also varied between

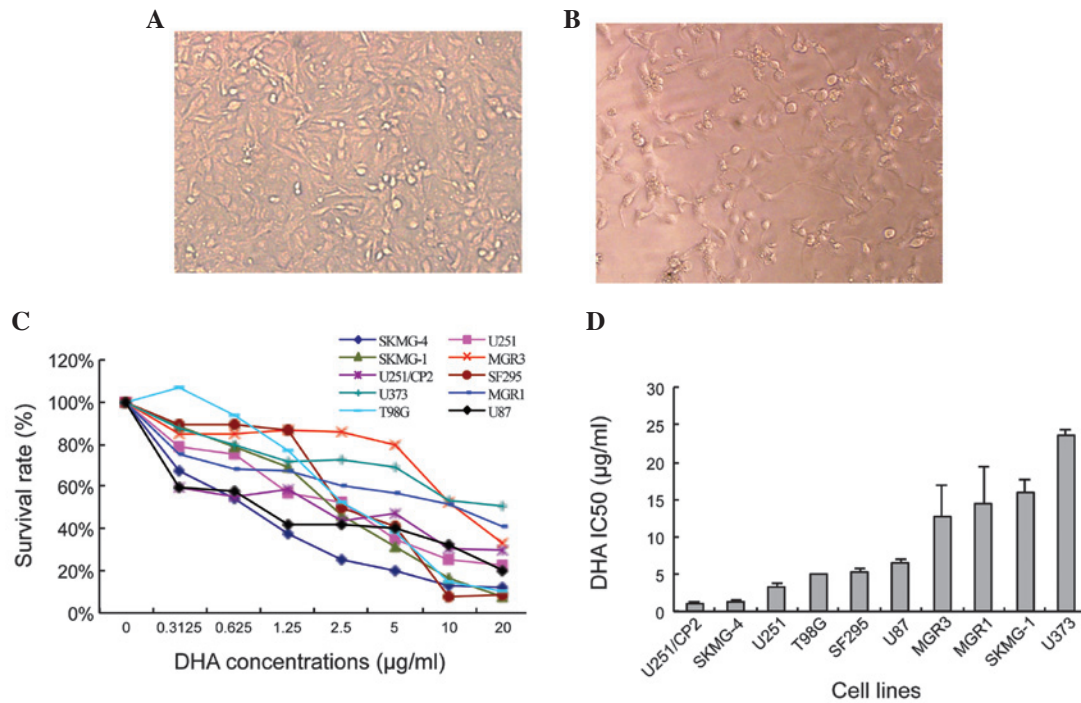


Figure 1. DHA exhibits cytotoxicity in glioma cell lines *in vitro*. The density and cellular morphology of SKMG-4 cells cultured (A) without and (B) with DHA (magnification, x200). The cells appeared lower in density and shrunken following treatment with DHA. (C) Survival curve of the 10 human glioma cell lines following treatment with serial concentrations of DHA for 72 h. (D) IC₅₀ of the 10 glioma cell lines. Values are expressed as the mean \pm standard deviation. DHA, dihydroartemisinin; IC₅₀, half maximal inhibitory concentration.

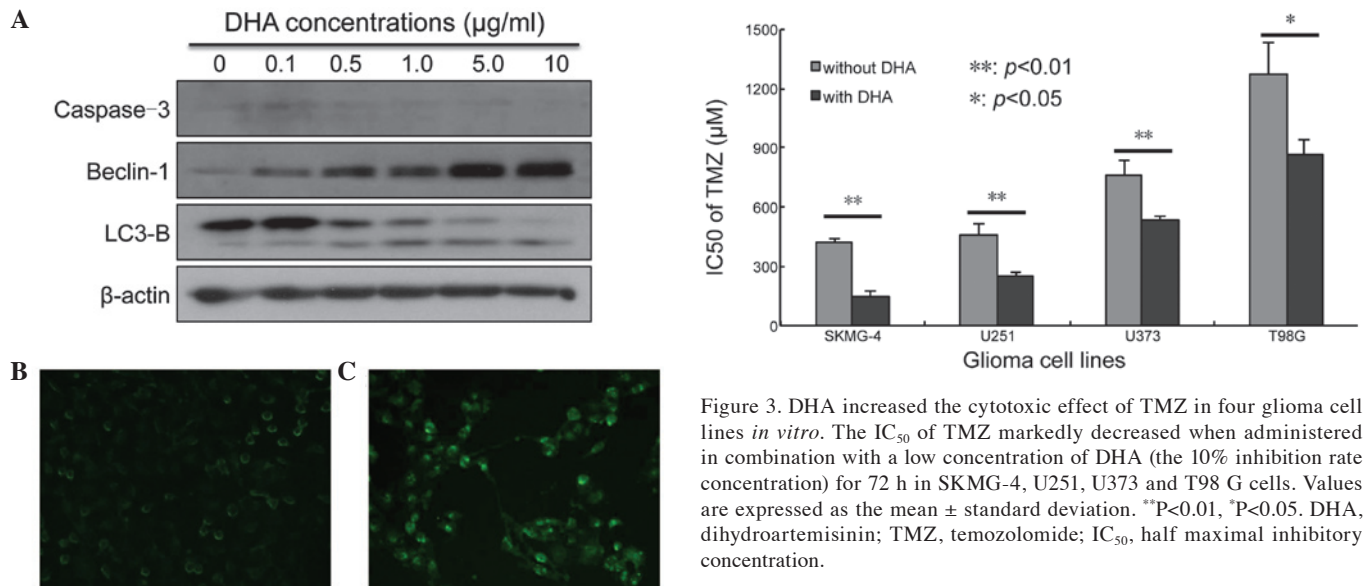


Figure 2. DHA activates autophagy in glioma cells. (A) The expression of the apoptosis molecular marker, Caspase-3, did not change following DHA treatment. However, the expression of autophagy markers Beclin-1 and LC3-B was markedly increased. (B) Monodansylcadaverine staining revealing that a 24-h treatment with 2 µg/ml DHA increased the formation of autophagic vacuoles in SKMG-4 cells compared with (C) the non-treated control cells (magnification, x200). DHA, dihydroartemisinin.

the ten human glioma cell lines, from 1.17 ± 0.08 µg/ml in U251/CP2 cells to 23.57 ± 0.80 µg/ml in U373 cells (Fig. 1D).

DHA activates autophagy, rather than apoptosis, in SKMG-4 cells. The cell lysates were collected from

Figure 3. DHA increased the cytotoxic effect of TMZ in four glioma cell lines *in vitro*. The IC₅₀ of TMZ markedly decreased when administered in combination with a low concentration of DHA (the 10% inhibition rate concentration) for 72 h in SKMG-4, U251, U373 and T98 G cells. Values are expressed as the mean \pm standard deviation. **P<0.01, *P<0.05. DHA, dihydroartemisinin; TMZ, temozolomide; IC₅₀, half maximal inhibitory concentration.

SKMG-4 cells following treatment with DHA for 72 h. Markers of apoptosis and autophagy were then detected by western blotting. There were no significant alterations to the expression of Caspase-3 following DHA treatment. However, the expression of the autophagy markers, Beclin-1 and LC3-B, increased upon DHA treatment in a dose-dependent manner (Fig. 2A). The autofluorescent reagent MDC, a specific autophagic vacuole marker, was also applied in order to monitor the process of autophagy. Following culture with DHA and MDC, the SKMG-4 cells were fixed and observed under a fluorescent microscope. It was revealed that MDC accumulated in DHA-treated cells, but not in untreated

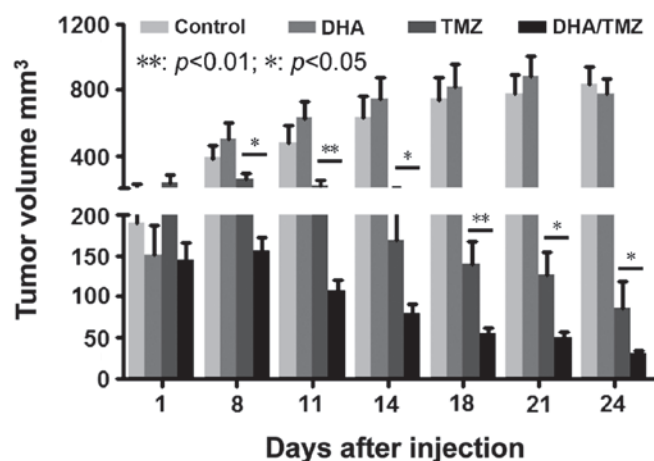


Figure 4. DHA enhances the tumor inhibition effect of TMZ *in vivo*. Treatment with 50 mg/kg TMZ reduced tumor formation compared with the control (dimethyl sulfoxide) group. Although 50 mg/kg DHA alone had little effect on the tumor size of nude mice, it enhanced the action of TMZ in decreasing tumor burden, compared with the TMZ alone-treated group. Values are expressed as the mean \pm standard deviation. ** $P<0.01$, * $P<0.05$. DHA, dihydroartemisinin; TMZ, temozolomide.

control cells (Fig. 2B and C). The data therefore indicated that autophagy was induced by DHA in glioma cell lines.

DHA promotes the cytotoxicity of TMZ in glioma cells. The present study aimed to investigate whether combined treatment with DHA and TMZ was able to increase cell sensitivity to TMZ. In total, four glioma cell lines, namely SKMG-4, U251, U373 and T98G, were used for the combination treatment due to the differences in their sensitivity to DHA and their genetic background. The 10% inhibition concentration of TMZ (IC_{10}) was calculated according to the results of the cytotoxicity assay (Fig. 1C). The concentration of DHA was fixed to the IC_{10} of each cell line and then combined with serial concentrations of TMZ. The IC_{50} of TMZ was calculated following co-treatment for 72 h. It was revealed that the application of DHA reduced the IC_{50} of TMZ in all four cell lines; from 417.36 ± 17.92 to 143.90 ± 28.74 μ M in SKMG-4 cells ($P<0.01$), 455.06 ± 64.39 to 246.84 ± 26.14 μ M in U251 cells ($P<0.01$), 759.03 ± 76.96 to 537.15 ± 11.88 μ M in U373 cells ($P<0.01$) and 1274.72 ± 159.70 to 863.20 ± 73.26 μ M in T98G cells ($P<0.05$) (Fig. 3). These data indicated that DHA sensitized glioma cell lines to TMZ.

DHA strengthens the tumor inhibitory effect of TMZ. The glioma U251 cell line was used for the *in vivo* tumor formation and inhibition assay, due to its ability to form tumors in xenografts *in vivo* and its sensitivity to DHA (low IC_{50}) *in vitro*. The tumor size was monitored every 3 days, following continuous treatment for 5 days with DMSO, DHA, TMZ and DHA+TMZ. It was revealed that 50 mg/kg DHA alone had no effect on tumor growth *in vivo* compared with the DMSO-treated control cells. By contrast, TMZ significantly suppressed tumor growth compared with that of the DMSO-treated control cells. Co-treatment with DHA and TMZ significantly reduced the tumor size compared with the effects of TMZ alone (Fig. 4; $P<0.01$, $P<0.05$). This indicated that DHA was able to strengthen the tumor inhibitory effect of TMZ *in vivo*. Taken together, the data demonstrated that DHA inhibited tumor cell proliferation

and sensitized glioma cells to TMZ *in vitro* and *in vivo*, potentially through the activation of autophagy.

Discussion

Glioma is the most common primary malignant brain tumor amongst adults. The median survival time of patients with high-grade glioma is generally <12 months, even with surgical resection, radiotherapy and chemotherapy (21-23). Therefore, the identification of novel, effective treatment strategies is necessary. Adjuvant chemotherapeutic reagents, as well as various combination therapies, have been extensively studied. Drugs used for combination therapies usually have clear pharmacokinetics, which leads to improved therapeutic efficiencies and tolerance, and reduces drug resistance and side effects (19,24,25).

Due to their efficiency and few side effects, traditional Chinese medicines have been extensively studied in anti-cancer research. Artemisinin is a Chinese herb derived from the plant, *Artemisia annua* L., and has been used in the treatment of fevers for $>1,000$ years (26). Rasheed *et al* (27) reported that artesunate, a derivative of artemisinin, was able to inhibit the proliferation and metastasis of non-small cell lung carcinomas in a chicken embryo model. A further study, which included 55 cancer cell lines, found that artesunate demonstrated anti-tumor activities in several types of tumor cell, including leukemia, as well as breast, ovarian, cervical, stomach, liver, pancreatic, colon and lung cancer (28). Artemisinin was therefore enrolled by the NCI in their drug-screening program. Previous data has suggested that artemisinin may be involved in multiple pathways. Screening approaches have established that artemisinin is involved in the inhibition of proliferation, the promotion of apoptosis and the inhibition of angiogenesis (28). Further studies have revealed that artemisinin contributes to the generation of ROS, which leads to heme-mediated endoperoxide cleavage, DNA damage and apoptosis (29). In addition, it has been found that artemisinin enhances the sensitivity of glioma cells to β , γ -rays (17).

As the major active metabolite of artemisinin, DHA has advantages over the original compound. The anti-cancer effects of DHA have been previously reported in several types of cancer, but not glioma. The present study identified that DHA mildly inhibited the growth of glioma cells. It was hypothesized that the inhibition of proliferation exerted by DHA may be cell-type specific, since its efficiency in the 10 human glioma cell lines evaluated was varied.

The findings of the present study indicated that the mechanisms underlying DHA-induced proliferation arrest may involve autophagy activation, since the expression of autophagic molecular markers increased following DHA treatment. Autophagy is a self-digestive process that degrades intracellular structures in response to oxidative stresses, ultimately leading to cell survival (30). However, cells die following prolonged autophagic activity. It has been established that cancer cells produce higher levels of ROS than those of normal cells, as a result of metabolic stress and proliferative requirements. A previous study reported that DHA induced autophagy in the mitochondria of leukemia K562 cells (31). A further study found that the sensitivity of cancer cells to artemisinin was attenuated following the over-expression of oxidative stress associated enzymes (32). The detailed mechanisms underlying the role of DHA in glioma cell autophagy will be investigated in future studies.

TMZ is an alkylating agent, whose therapeutic benefit depends upon its ability to alkylate DNA at the N⁷ or O⁶ positions of guanine residues. The low cytotoxicity, high lipid solubility and small molecular weight of TMZ facilitates its passage through the blood-brain barrier. These properties established TMZ as an effective, first-line chemotherapeutic drug for glioma patients. Kanzawa *et al* (33) identified that TMZ cytotoxicity resulted from the induction of autophagy, rather than apoptosis, in malignant glioma cells as the blockage of autophagy significantly decreased the anti-tumor effect of TMZ. The results of the present study revealed that DHA interacted with TMZ to enhance the anti-cancer effect *in vitro* and *in vivo*.

In conclusion, DHA may inhibit the growth of glioma cells through the induction of autophagy. Furthermore, cell response to TMZ may be enhanced significantly by co-treatment of DHA, both *in vitro* and *in vivo*. However, the mechanisms underlying the interaction between DHA and TMZ in autophagy and other signaling pathways require further investigation.

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