3BDO Inhibits proliferation, epithelial–mesenchymal transition (EMT) and stemness via suppressing survivin in human glioblastoma cells

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Primary research

Keywords: 3BDO, glioblastoma, EMT, GSC, survivin

Posted Date: February 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-179269/v1>

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Abstract

Background

Glioblastoma (GBM) is a tumor of the central nervous system carries an extremely poor prognosis. Unfortunately, it also is the most frequently encountered tumor in this region. These tumors arise from glioblastoma stem cells (GSCs), which are glioma cells that are known to possess high degrees of stemness. GBM invades through the process of EMT, which features loss of cell differentiation and polarity. Survivin is a type of apoptotic inhibitor that has been characterized in several malignancies such as glioma. Normal tissues rarely express survivin. On the other hand, 3-benzyl-5-((2-nitrophenoxy) methyl) dihydrofuran-2(3H)-one (3BDO) represents an autophagy inhibitor and activates the mTOR pathway. It has been reported that 3BDO shows anti-cancer activities in lung carcinoma. However, the effects of 3BDO on GBM remains unknown. Therefore, the purpose of this study was to explore the role and molecular mechanisms that 3BDO mediates in GBM.

Method

CCK-8 experiments and clone formation assay were performed to detect the cell proliferation. Transwell assay was conducted to examine cell migration and invasion. Western blotting and immunofluorescence staining was used to analyze protein expression levels. Xenograft mouse model was used to evaluate the effect of 3BDO *in vivo*.

Results

We found that 3BDO inhibited U87 and U251 cell proliferation in a dose-dependent manner. Additionally, 3BDO decreased the sphere formation and stemness markers (sox2, nestin and CD133) in GSCs. 3BDO also inhibited migration, invasion and suppressed EMT markers (N-cadherin, vimentin and snail) in GBM cells. Moreover, we found that 3BDO downregulated survivin expression of survivin both in GBM cells (U87, U251) and GSCs. Furthermore, overexpression of survivin reduced the therapeutic effects of 3BDO on GBM cell EMT, invasion, migration and proliferation, as well as decreased stemness in GSCs. Finally, we demonstrated that 3BDO inhibited tumor growth in a tumor xenograft mouse model constructed using U87 cells. Similar to the *in vitro* findings, 3BDO diminished survivin expression, stemness and levels of EMT markers *in vivo*.

Conclusions

Our results demonstrated that 3BDO repressed GBM via downregulating survivin-mediated stemness and EMT both *in vitro* and *in vivo*.

Background

Glioblastoma (GBM) is a high-grade glioma which features high rates of invasion and rapid growth. Despite the innovation of surgical and medical treatment modalities, patients diagnosed with GBM rarely

survive longer than 15 months upon diagnosis [1, 2]. Currently, temozolomide (TMZ) is the only one first-line agent for GBM chemotherapy, but its effectiveness has been marred by drug resistance. More useful agents are urgently needed to manage GBM.

Cancer recurrence and metastasis are often attributed to cancer stem-like cells (CSCs) given their capabilities to regenerate tumors by resisting standard chemo- and radiotherapy regimens [3–5]. A myriad of cancers has been shown to harbour CSCs, including GBM. CSCs in GBM are referred to as glioma stem cells (GSCs), which have been documented to be able to carry out multi-lineage differentiation, self-renew and extensively proliferate. Current research suggests that these GSCs may be a pivotal molecule in GBM growth, with its elimination potentially inhibiting GBM growth [6–8]. It is therefore of great interest to explore the potential of GSCs as a mode of GBM treatment.

Epithelial-mesenchymal transition (EMT) represents a transition where the normal epithelial cell acquires a mesenchymal phenotype. The crux of cancer lies in cells undergoing EMT, which increases the motility of individual cancer cells and allows them to invade across epithelial junctions and extracellular matrices [9, 10]. EMT has a strongly cemented position in GBM. Both *in vivo* and *in vitro* studies on GBM show that activation of an EMT-like program results in enhanced malignant migration and invasion [11, 12]. Additionally, patients with aggressive GBM and a poor prognosis invariably demonstrate elevated levels of mesenchymal markers such as N-cadherin, snail and vimentin [13, 14]. Therefore, EMT inhibition appears to be a feasible modality in treating GBM.

Survivin is a protein that is associated to apoptosis inhibition and is also known as BIRC5 or baculoviral inhibitor of apoptosis repeat-containing 5. It is significantly increased in multiple cancers such as colon cancer, lung cancer, breast cancer and melanoma [15, 16]. Similarly, increasing studies have found significantly raised survivin expression levels in glioma cells in comparison to normal central nervous tissue along with a positive association between survivin expression and glioma pathological grade. Patients with higher levels of survivin have been documented to possess a worse prognosis [17]. In addition, increased survivin expression promoted EMT and the stemness process [16, 18].

3-Benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2 (3H)-one (3BDO) is an mTOR activator that works as an autophagy inhibitor. For example, it has been reported that 3BDO exposure significantly diminishes the amount of autophagosomes in APP/PS1 transgenic mice while simultaneously improving the subjects' memories [19]. In addition, 3BDO partially reversed the maturation of Rheb1-deficient neutrophils by mTOR inactivation [20]. Furthermore, accumulating evidence demonstrated that 3BDO may be an independently acting mTOR modulator. For instance, 3BDO alleviated plaque endothelial cell death and slowed down the establishment of atherosclerosis in mice, but in a manner that was not dependent on autophagy or mTOR activity [21]. 3BDO has also been demonstrated as an *in vivo* and *in vitro* inflammatory suppressor [22]. Furthermore, 3BDO has also been shown to inhibit cancer cells growth when administered in combination with DPB [23]. It remains to be discovered if 3BDO exerts potential anti-cancer properties in GBM.

This study first dissects the impact of 3BDO on human GBM in vitro, followed by experiments specifically evaluating EMT and stemness properties in association with survivin expression. 3BDO was also investigated on tumor xenograft mice models, and was found to be able to inhibit tumor growth. This study not only identifies 3BDO as a potential agent but also laid the theoretical foundation for a novel approach for GBM treatment.

Method And Materials

Chemicals, Reagents and Antibodies

3BDO was obtained from Selleck. DMSO was used to produce a 3BDO suspension which was kept at 4°C. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were procured from (Grand Island, USA). Antibodies against N-cadherin, nestin, CD133, vimentin, GAPDH and survivin were bought from Cell Signaling Technology (Beverly, MA). Abcam (Cambridge, MA) provided antibodies against snail and sox2.

Cell Culture

The glioblastoma cell lines U87 and U251 were obtained from the Chinese Academy of Medical Sciences (Beijing, China). All cell lines were maintained in DMEM supplemented with 10% FBS. GSCs used in this study was obtained with thanks to Dr Jia Ouyang from Soochow university. These cells were cultured in neurobasal medium containing 20ng/ml basic fibroblast growth factor (bFGF) (Peprotech, USA), 2% B27 (Gibco, USA), 1% Glutamine (Gibco, USA) and 20ng/ml epidermal growth factor (EGF) (Peprotech, USA). All above cells were incubated in a humidified atmosphere at 5% CO₂ at 37°C.

Cell Viability Assay

A 96-well plate was used to contain cells at a density of 4×10^3 cells/well for 24 h. Each well was exposed to various 3BDO concentrations. At the end of the treatment period, cells were further treated with 10 µl of the CCK-8 solution and allowed to incubate for an hour at 37°C. A microplate reader was then used to assess cell viability.

Clonogenic assay

Cells were suspended in DMEM medium with 10% FBS. A 6cm diameter plate was used to house 500 cells. The selected 3BDO concentration was then added into the plate and left to culture for 1 week at 37°C in 5% CO₂ atmosphere until obvious cell colonies were seen. The cells were then rinsed thrice with PBS before they were subjected to fixation with methyl alcohol and stained with crystal violet for 10 min. Colonies were counted after three final rinses with PBS.

Cell migration and invasion Assay

Corning supplied the Transwell system used to study cell invasion and migration in this study (Corning, USA). The upper chamber was used to house the cells (2×10^4 suspended 200 µl DMEM supplemented

with 1% FBS). Chambers were coated either with or without 100 µl matrigel (BD Biosciences, CA, USA) to conduct the migration or invasion assay, respectively. 600 µl DMEM supplemented with 20% FBS was placed in the lower chamber along with various 3BDO concentrations. The system was left alone for 24 hours. After this period, ethanol was used to fix cells in the lower chamber before they were stained with 0.1% crystal violet in methanol. Cells were counted in three random fields captured at 100x magnification.

Transfection

Both GCSs and the GBM cell lines were transfected with survivin gene-containing plasmid in order to produce cells that overexpressed survivin (GeneCopoeia, Maryland Rockville, USA). Control cells were transfected with the vector only. All experiments were conducted in compliance to instructions stipulated by the manufacturer.

Western Blot

Xenograft glioblastoma tissue homogenates and cell lysates were used for Western blot experiments. The Pro-prep TM protein Extraction Solution (iNtRON Biotechnology, Korea) was used to extract protein. A 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then used to separate component proteins before blotting them onto polyvinylidene difluoride membranes (Merck, KGaA, Darmstadt, Germany). 5% BSA was used to block endogenous reactions for an hour under room temperature. The membranes were then incubated overnight with specific primary antibodies at 4°C. Horseradish protein-conjugated secondary antibodies were then exposed to the membranes for another hour at room temperature. The Super Signal ECL (Pierce, Rockford, IL, USA) system was used to interpret the results.

Immunofluorescence

After the indicated treatment, GSCs were incubated on cell climbing slices coated with polylysine for 1 hour. This was followed by immunofluorescence staining in accordance to standard protocols. 1× PBS was first used to rinse the slides thrice before normal goat serum supplemented with 0.3% Triton X-100 for an hour. Primary antibodies suspended in 0.01M PBS were added to the slides and allowed to incubate overnight at 4°C. Cells were again washed thrice with PBS the next morning and exposed for 45 minutes to Alexa Fluor 555-conjugated secondary antibody at room temperature. Slides were again rinsed thrice and counterstained by DAPI for 10 min, washed and air-dried before imaging. Images were observed and acquired with either light or fluorescence microscopy under a confocal microscope.

U87 Xenograft Mouse Model with 3BDO Treatment

Female BALB/c nude mice were procured from the Animal Experiment Center of Southern Medical University (Guangzhou, China). Mice were between the ages of 6–8 weeks and reared under carefully established protocols by the Institutional Animal Care of the Second Affiliated Hospital of Guangzhou Medical University. Approval for experimental procedures using animals was granted by the Institutional Animal Care Committee of the Institutional Animal Care of the Second Affiliated Hospital of Guangzhou Medical University and guidelines for the ethical review of laboratory animal welfare (People's Republic of

China National Standard GB/T 35892) were followed. All mice received subcutaneous injections to the dorsum containing cultured U87 cells (5×10^6 cells per mouse). Calipers were used to assess tumor size in two orthogonal directions. Tumor volume (mm^3) was derived based on the formula: $1/2 \times \text{length} \times \text{width}^2$. Upon achieving tumor size of approximately 150mm^3 , mice were either orally fed with vehicle or 3BDO (80 mg/kg/day) ($n = 5$ mice per group). Mice body weights and tumor sizes were assessed once every 5 days. All tumors were harvested for further analysis after mice were sacrificed at the end of these experiments.

Statistical analysis

All data are composites of three repeated experiments and were analysed using the SPSS 20.0 software. An independent T-test was used to perform simple comparisons between 2 groups and multiple comparisons between the groups were evaluated using one-way analysis of variance, followed by post hoc analyses, which were carried out using Dunnett's T3 test or Turkey test. $P < 0.05$ was interpreted as achieving statistical significance.

Results

3BDO inhibits proliferation, migration and invasion in GBM cells

To confirm whether 3BDO regulates the proliferation of GBM cells, we carried out a CCK-8 assay in U87 and U251 cells. Figure 1A-B demonstrates that U87 and U251 cell growth were inhibited by 3BDO in a dose-dependent manner after 24 hours of treatment.

Subsequently, we conducted a colony formation assay to further investigate the effects of 3BDO on cell growth in U87 and U251 cells. Similarly, 3BDO also suppressed the ability for colony formation in a dose-dependent way (Fig. 1C-D).

Next, we observed the invasive and migratory abilities of cells treated with 3BDO using a Transwell assay. 3BDO treatment resulted in lower rates of cell invasion and migration in contrast to untreated cells (Fig. 1E-H). These *in vitro* findings strongly suggest that GBM cell proliferation, invasion and migration are hindered by 3BDO.

3BDO decreased the EMT markers and inhibited survivin expression in GBM cells

We proceeded to investigate how EMT marker expressions (N-cadherin, vimentin and snail) were altered by 3BDO. Exposure to this molecule resulted in cells expressing lower levels of N-cadherin, vimentin and snail (Fig. 1I). Moreover, 3BDO decreased survivin expression levels in a dose-dependent manner in U87 and U251 cells. Our findings indicated that 3BDO decreased the EMT markers and inhibited survivin expression in GBM cells.

3BDO inhibits cell growth and downregulated survivin as well as the stemness markers in GSCs

The initiation and progression of GBM have been attributed to GSCs. It is therefore of sound scientific reasoning to pursue GSC as a target for treating GBM. In this study, we used a patient-derived GSC to perform the experiment. The GSCs were cultured in a sphere-forming medium and were treated with different concentrations 3BDO for 72 hours. The number of spheres were quantified at the end of this period. As showed in the Fig. 2A-B, 3BDO inhibited the number of spheres in a dose-dependent manner.

Sox2 is an important marker of GSC stemness. We used cell immunofluorescence experiments to examine the protein expression level of sox2 and found that the sox2 expression level decreased with decreasing 3BDO treatment, and vice versa (Fig. 2C-D). Next, western blots were done to quantify levels of stemness markers such as nestin, CD133 and sox2. The results showed that the aforementioned stemness markers expression level were downregulated after being incubated with 3BDO for 24 hours. Moreover, we found 3BDO decreased survivin expression levels in a dose-dependent manner (Fig. 2E). These results also suggested that 3BDO was able to suppress GSCx stemness while downregulating surviving levels in GSCs.

Overexpression of survivin decreased the effects of 3BDO in GBM cells and GSCs

Previous studies have highlighted the role of surviving in EMT. We sought to confirm if 3BDO-induced EMT suppression was mediated by survivin suppression. U87 and U251 cells were artificially induced to overexpress survivin before being subjected to a 24 hour incubation period with either 3BDO or vehicle. GBM cells proliferation, migration and invasion were raised in the presence of surviving overexpression (Fig. 3A-F). We also found that upregulation of survivin increased EMT markers expression including N-cadherin, vimentin and snail (Fig. 3G). Furthermore, we found that the overexpression of survivin reduced the above inhibitory effects on GBM cells induced by 3BDO.

Multiple studies reported that survivin was expressed abundantly in GBM tissues, and also in human-derived GSC cultures. Survivin downregulation could abolish both *in vivo* and *in vitro* GSCs growth. To further investigate whether 3BDO inhibited GSCs through its effect on survivin, we overexpressed survivin plasmid or its vector plasmid followed by incubating the cells in the presence or absence of 3BDO. We found that overexpression of survivin enhanced the number of sphere formation and levels of stemness markers such as nestin, CD133 and sox2 expression. Moreover, overexpression of survivin reduced the effects of 3BDO on sphere formation and stemness markers upregulation in GSCs (Fig. 4).

These results suggest that 3BDO exerts an anti-tumor role by suppressing survivin-triggered EMT in GBM cells and exerting an anti-stemness effect in GSCs.

3BDO suppressed GBM growth in a U87 xenograft mouse model

A U87 xenograft mouse model was used to explore the *in vivo* effect of 3BDO on tumor growth. A 25-day long 3BDO treatment protocol resulted in significantly lower tumor weights and volumes (Fig. 5A-C), without affecting mice body weights (Fig. 5D) in contrast to untreated groups. Additionally, we confirmed that the endogenous survivin, snail, N-cadherin, vimentin and GSC markers such as nestin, sox2 and CD133 in tumors dissected from the U87 xenograft mice was suppressed by 3BDO (Fig. 5E).

These findings are in agreement with our *in vitro* findings, suggesting that 3BDO suppressed tumor growth and abolished GSC stemness in glioblastoma xenograft models, thereby highlighting its potential as a therapeutic GBM candidate.

Discussion

Despite the advent of curative GBM therapy that involves a combination of surgical resection and chemoradiotherapy, GBM patient prognosis is still abysmal due to its tendency to regrow and aggressively invade. It is vital that newer treatment agents are investigated. This study presents 3BDO, a known mTOR activator, as a likely EMT suppressor in GSC cells that works through survivin downregulation.

3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO), a novel mTOR activator, has demonstrated a variety of biological activity in addition to autophagy suppression [24, 25]. It has been reported that 3BDO could improve the cognition defects by regulating autophagy in APP/PS1 AD mice models [19]. In addition, 3BDO may be beneficial in treating cardiovascular diseases by suppressing autophagy [26]. Moreover, 3BDO could inhibit both *in vivo* and *in vitro* production of inflammatory cytokines [22]. However, the effects of 3BDO in cancer are seldom reported. Zhao Y et al showed that 3BDO combined with DPB could promote more effective apoptosis in A549 cells [23]. The current study explores for the first time the inhibitory effects of 3BDO on GBM as well as in GSC cells.

EMT, as a proven mechanism in the process of tumor development. It works by maintaining tumor stemness, enhancing drug resistance and enhancing cellular invasiveness – all of which greatly promote tumor growth [27]. GSCs are infamous for their high levels of resistance towards chemoradiotherapy as well as a strong ability to initiate tumor growth [6, 8]. Therefore, it is essential to find new treatments that inhibit EMT and GSCs to cure GBM. Several agents have been identified as EMT suppressors which in turn inhibit GSCs. One such potential agent is 3BDO, however, little is known regarding its effect on EMT and GSC in GBM. In the current study, we proved that 3BDO possessed anti-invasive and anti-migratory properties which may reverse EMT progression in GBM. Moreover, we demonstrated that 3BDO inhibited GSC cell growth by suppressing stemness.

Survivin has been demonstrated to be significantly upregulated in GBM and may play a role in promoting GBM development. GBM patients with higher survivin expression had shorter survival times in contrast to patients with lower surviving expression. In addition, EMT regulation has been reported to be mediated by survivin in GBM.

Liu F et al reported that IGF-1 triggered EMT in hepatocellular carcinoma by activating surviving [28]. Additionally, Lee J et al showed that TGF- β regulated the EMT process by upregulating surviving [29]. It has been reported that silencing of survivin expression could suppress EMT activation to decrease the invasive and migratory abilities in HCC cells [30]. YM155 was able to inhibit migration and invasion through depressing survivin-mediated EMT in U87 and U251 cells [31]. Furthermore, Guvenc H et al reported that suppression of survivin could impair GSCs survival [18]. Moreover, brexpiprazole has been shown to promote GSC sensitivity to chemotherapy drugs by down-regulating surviving [32]. Therefore, targeting survivin is a promising strategy in the management of GBM. 3BDO, an autophagy inhibitor, recently also showed that it could regulate TGFB2 in a manner that does not activate the autophagy process [22]. In our study, we found that 3BDO could inhibit GBM and GSCs via downregulation of survivin. Moreover, GBM cells and GSCs that overexpress survivin appear to develop 3BDO resistance. This suggests that surviving may be a target of 3BDO.

Most agents that exert an anti-GBM effect *in vitro* remain ineffective *in vivo*. Previous drug candidates that have been shown to inhibit tumor growth in a subcutaneous GBM model failed to suppress GBM in the CNS due to the existence of the blood-brain barrier (BBB). Moreover, safety and efficacy profiles have to be considered, especially in the development of an anti-cancer agent. In previous investigations, 3BDO has been applied in *in vivo* experiments including in CNS and showed a good safety profile [25, 33]. We found that 3BDO suppressed GBM growth in a subcutaneous GBM model in a manner similar to that demonstrated in our *in vitro* experiments. The lack of significant changes in overall mice body weights confirmed the presence of drug tolerance.

Overall, we proved that 3BDO successfully suppressed EMT and stemness in GBM both *in vivo* and *in vitro*. Additionally, we identified survivin as a potential target of 3BDO. We also demonstrated, for the first time, that 3BDO exerted anti-GBM properties in a subcutaneous glioma model, which was consistent with its mechanism of action demonstrated in the *in vitro* experiments. Therefore, 3BDO represents a potential therapeutic drug for GBM treatment.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (81901117), Natural Science Foundation of Guangdong Province (2019A1515010926).

Authors' contributions

Yezhong Wang, Zhou Xing and Yunxiang Ji designed the study; Zhaotao Wang, Yongping Li, Minyi Liu and Danmin Chen performed the experiments and prepared the figures; Zhaotao Wang and Yongping Li contributed to drafting the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

Figure 1

3BDO diminished invasion, migration and proliferation as well as the expression of EMT-associated molecules and surviving in GBM cells (A-B) 3BDO was used to treat U87 and U251 cells for 24 hours followed by tests to confirm cell viability (C-D) 3BDO was administered for 7 days to U87 and U251 cells prior to performing the clonogenic assay. (E-H) GBM cells were incubated with selected 3BDO concentrations for 24 hours and were subjected to a Transwell assay to evaluate the degree of cell

invasion and migration (I) Western blot was used to investigate survivin and EMT-associated proteins in U87 and U251 cells exposed to selected 3BDO concentrations for 24. Each group consisted of n=3 or 4 and all investigations were performed thrice. *P < 0.05 compared with the control (0 μ M).

Figure 2

3BDO inhibits cell growth and downregulated survivin as well as the stemness markers in GSCs. GSCs were cultured with selected doses of 3BDO for 72 hours and were then subjected to (A-B) quantification of spheres formed (C-D) immunofluorescence examination to determine sox2 expression levels (E) Western blotting to determine protein expressions of stemness marker. Each group consisted of n=3 or 4 and all investigations were performed thrice. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control (0 μ M).

Figure 3

Overexpression of survivin reduced the inhibition of cell proliferation, migration, invasion and EMT induced by 3BDO. (A-B) GBM cells were transfected with survivin or the vector plasmid for 12 hours before being transferred to 96-well plates and treated with 100 μ M 3BDO or the vehicle for 24 hours. Cellular proliferation was evaluated with the CCK-8 assay. (C-F) Migration and invasion assays were performed on transfected GBM cells using a Transwell assay (G) GBM cells transfected with survivin or the vector

plasmid were then treated with 100 μ M 3BDO for 24 hours. Western blotting analysis was conducted to detect protein expression. *P < 0.05 vs control; #P < 0.05 compared with either 3BDO incubation or survivin transfection alone.

Figure 4

Overexpression of survivin decreased the impact of 3BDO in GSCs. GSCs transfected with survivin or the vector plasmid for 12 hours were cultured with the 100 μ M 3BDO. Additionally, GSCs were incubated with or without 100 μ M 3BDO for 72 hours and were then subjected to (A-B) quantification of spheres formed (C-D) immunofluorescence examination to determine sox2 expression levels (E) Western blotting to determine protein expressions of stemness marker. Each group consisted of n=3 or 4 and all investigations were performed thrice. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control (0 μ M).

Figure 5

3BDO decreased glioma growth in xenograft mouse model. (A) suppression in the size of the xenografted U87 tumors were photographed. The tumor volume (B), tumor weight (C) and body weight (D) were measured per 5 days. (E) At the end of the experiments, tumor tissues were excised from the mice, and then western blot was used to detect the protein expression. n=3 or 4 and all tests were performed in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control (0 μ M).