Inhibition of monocarboxylate transporter 1 suppresses the proliferation of glioblastoma stem cells

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Abstract Recent evidence suggests that a minor subset of cancer cells, termed cancer stem cells (CSCs), have self-renewal and tumorigenic potential. Therefore, the characterization of CSCs is important for developing therapeutic strategies against cancer. Cancer cells rely on anaerobic glycolysis to produce ATP even under normoxic conditions, resulting in the generation of excess acidic substances. Cancer cells maintain a weakly alkaline intracellular pH to support functions. Glioblastoma is an aggressive malignancy with a poor 5-year survival rate. Based on the hypothesis that ion transport-related molecules regulate the viability and function of CSCs, we investigated the expression of ion transport-related molecules in glioblastoma CSCs (GSCs). Quantitative RT-PCR analysis showed that monocarboxylate transporter1 (MCT1) were upregulated in GSCs, and inhibition of MCT1 decreased the viability of GSCs compared with that of non-GSCs. Our findings indicate that MCT1 is involved in the maintenance of GSCs and is a promising therapeutic target for glioblastoma.

Keywords Glioblastoma · Cancer stem cell · Hypoxia · Monocarboxylate transporter · Lactic acid · Carbonic anhydrase

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ATP          | Adenosine triphosphate |
| b-FGF        | Basic fibroblast growth factor |
| CA           | Carbonic anhydrase |
| CSC          | Cancer stem cell |
| DMEM         | Dulbecco’s modified Eagle’s medium |
| DMSO         | Dimethyl sulfoxide |
| EGF          | Epidermal growth factor |
| FBS          | Fetal bovine serum |
| GSC          | Glioblastoma cancer stem cell |
| HA           | Hypoxia-adapted |
| MCT          | Monocarboxylate transporter |
| NHE          | Na+–H+ exchanger |
| PC/SM        | Penicillin/streptomycin |
| pHMBS        | p-hydroxymercuribenzoic acid sodium salt |
| qRT-PCR      | Quantitative reverse transcription polymerase chain reaction |
| RT           | Room temperature |
| UPL          | Universal probe library |

Introduction

A subpopulation of tumor cells with the properties of stem cells, including self-replication ability and pluripotency, have been identified and termed cancer stem cells (CSCs). The CSC hypothesis states that tumors show a hierarchical organization based on the supply of cells from CSCs. Furthermore, CSCs are resistant to current anticancer drugs and radiation and play an important role in metastasis [1–4].

Glioma is the most common primary brain tumor. Glioblastoma is an aggressive form of this tumor and has a
poor prognosis, with a 5-year survival rate of ≤5 % [5]. A cell population known as glioblastoma stem cells (GSCs) is involved in the maintenance of glioblastomas [6, 7] as well as resistance to chemotherapies [8, 9]. Therefore, we have to develop novel molecular strategies against GSCs.

Cancer cells rely on anaerobic glycolysis for energy production even in the presence of oxygen in what is termed the Warburg effect [10, 11]. Glioblastoma cells produce ATP via the Warburg effect [12], resulting in the production of acidic substances, such as carbon dioxide and lactic acid, which accumulate as metabolites in the cytoplasm. Protons and metabolites associated with acidic substances are released by cancer cells to the extracellular space or accumulate in acidic granules, and cell function is preserved through the maintenance of a weakly alkaline intracellular environment. The acidity of the cancer microenvironment is thought to contribute to malignant progression and drug resistance [13]. Ion transport-related molecules, such as carbonic anhydrase (CA), Na\(^+\)–H\(^+\) exchanger (NHE), and monocarboxylate transporter (MCT), are responsible for this function and are expressed at higher levels in cancer cells than in normal cells [14–20].

Here, we hypothesized that enhanced anaerobic glycolysis in CSCs, which are abundant in the hypoxic environment, results in the production of excess acidic substances [21]. We therefore examined the expression and activity of ion transport-related molecules that release protons to the extracellular medium.

**Materials and methods**

**Cell culture**

The glioblastoma cell lines U-251 MG (mutant p53) and U-87 MG (wild-type p53) were purchased from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium (DMEM)/low glucose (Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (PC/SM; Wako) (DMEM/low) in a CO\(_2\) incubator [MCO-18AIC (UV); SANYO Electric, Osaka, Japan] with 5% CO\(_2\) at 37 °C for normoxia or in a O\(_2)/CO\(_2\) incubator (WKN-9200EX; Waken Btech, Kyoto, Japan) with 1% O\(_2\) and 5% CO\(_2\) at 37 °C for hypoxia. Hypoxia-adapted (HA) cells were those incubated for at least 1 month under hypoxic conditions. Cells were seeded at a density of 5.0 × 10\(^4\) cells/ml. We defined that non-CSCs normoxia were adherent cells incubated under normoxic conditions, and non-CSCs hypoxia were adherent cells incubated under hypoxic conditions.

**CSC preparation**

CSCs were prepared by the sphere formation assay, in which cells were cultured in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) containing 1% PC/SM, 2% B-27 [B-27\(^{®}\) Serum-Free Supplement (50×); Thermo Fisher Scientific], 20 ng/ml epidermal growth factor (EGF; Peprotech, Rocky Hill, NJ, USA), and 20 ng/ml basic fibroblast growth factor (b-FGF; Peprotech) for 7 days in ultra-low attachment 6-well plates (Corning International, Corning, NY, USA). EGF and b-FGF were added at 20 ng/ml on day 3. U-251 MG cells were seeded at a density of 1.0 × 10\(^5\) cells/ml. U-87 MG cells were seeded at a density of 6.7 × 10\(^4\) cells/ml. We defined that CSCs normoxia were spheres incubated under normoxic conditions.

**Immunofluorescence analysis**

Adherent cells (non-CSCs) were cultured in DMEM/low in glass-bottom microwell dishes (P35G-1.5-14-C; MatTek, Ashland, MA, USA) for 3 days. After culture, cells were fixed in 4% paraformaldehyde (PFA; Nacalai Tesque, Kyoto, Japan) for 30 min, and then incubated with primary antibodies (rabbit polyclonal anti-human Nanog, 1:500; Merck Millipore, Billerica, MA, USA; rabbit monoclonal anti-human Nestin, 1:100; Abcam, Cambridge, UK; and mouse monoclonal anti-human CD133/1, W6B3C1, 1:50; Miltenyi Biotec, Bergisch Gladbach, Germany) overnight at room temperature (RT). Cells were then incubated with secondary antibody (1:500) (Alexa Fluor 488 anti-rabbit IgG or Alexa Fluor 488 anti-mouse IgG; Thermo Fisher Scientific) for 2 h at 4 °C. Nuclei were stained with Hoechst33342 (1:10,000) (Thermo Fisher Scientific).

To produce sphere (CSCs) samples, cells were harvested and fixed in 50% methanol for 10 min at RT with shaking (60 rpm), and 2% PFA for 20 min at RT with shaking (60 rpm). After fixing, cells were incubated with primary antibody (same concentrations) overnight at RT with shaking (60 rpm), followed by incubation with secondary antibody (1:500) for 2 h at 4 °C with shaking (60 rpm). Cells were then suspended in mount medium (VECTORSHIELD; Vector Laboratories, Burlingame, CA, USA) and seeded on microscope slides with a cover glass (CoverWell™ Imaging Chambers PCI-A-0.5; Grace Bio-Labs, Bend, OR, USA).

For staining of hypoxic areas, cells were treated with pimonidazole HCl (200 μM) for 2 h before harvesting and then stained with anti-pimonidazole mouse IgG\(_1\) monoclonal antibody (clone 4.3.11.3) (1:50) (Hypoxyprobe™-1 Kit; Hypoxyprobe, Burlington, MA, USA). Samples were visualized using a confocal laser scanning microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany). ImageJ software was used for image analysis.
Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted with the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare UK, Little Chalfont, Bucks, UK) following the manufacturer’s protocol. RNA was quantified using NanoDrop 2000 (Thermo Fisher Scientific). Then, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer’s protocol. The concentration was adjusted to 20 ng/μl.

qRT-PCR was performed on a 7500 Real-Time PCR System (Thermo Fisher Scientific) with a FastStart Universal Probe Master (Rox) (Roche Applied Science, Penzberg, Germany). The relative expression of the selected genes was normalized to that of 18S rRNA for each sample. As a control, 5 ng of cDNA (total RNA substantial amount) was used per response. Templates were amplified under the following conditions: 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The combinations of various primer sets (Thermo Fisher Scientific) and probes (Roche Applied Science) are shown in Supplementary Table S1. The primer sequences and probes were determined using Universal Probe Library (UPL) (https://qpcr.probefinder.com/organism.jsp).

Inhibitor preparation

p-hydroxymercuribenzoic acid sodium salt (pHMBS; Sigma-Aldrich) was dissolved in sterile milliQ to a concentration 15 mM, and diluted with medium to achieve working concentrations. AR-C117977 was dissolved in dimethyl sulfoxide (DMSO; Wako) to a stock concentration of 100 mM, and diluted with medium to reach working concentrations. DMSO was used at a concentration of 0.2 %.

WST-8 assay

The cytostatic effect of MCT inhibitors (pHMBS, AR-C117977) on adherent cells was determined using the WST-8 assay. Cells were seeded at a density of 3.0 × 10^3 cells/90 μl per well in 96-well cell culture plates (Corning International) and treated with inhibitors (10 μl per well) at 24 h after seeding. After 72 h, the Cell Count Reagent SF (Nacalai Tesque) was added at 10 μl per well, and plate were placed in a CO₂ incubator for 3 h. Absorbance was measured at 450 nm (reference wavelength: 630 nm) with a microplate reader (Model 680 Microplate Reader; Bio-Rad, Hercules, CA, USA).

CellTiter-Glo® 3D cell viability assay

The cytostatic effect of MCT1 inhibitors on spheres was determined using the CellTiter-Glo® 3D cell viability assay. Cells were seeded at a density of 3.0 × 10^3 cells/90 μl per well in ultra-low attachment 96-well plates (Corning International) and treated with inhibitors (10 μl per well) at 24 h after seeding. After 6 days, the cell-containing media were transferred to a 96-well white plate (96F Nuncolon™ Delta White Microwell SI; Thermo Fisher Scientific). CellTiter-Glo® 3D Reagent (Promega, Madison, WI, USA) was added at 100 μl per well, mixed by shaking for 5 min, and incubated for 25 min at RT. Luminescence was measured with a luminometer (GloMax® Discover System; Promega).

Lactate assay

The cells were harvested and suspended in PBS, and solutions were disrupted to generate intracellular lactate samples using Handy Sonic (UR-20P; Tomy Seiko, Tokyo, Japan). A Lactate Assay Kit II (BioVision, Milpitas, CA, USA) was used for measurements following the manufacturer’s protocol. Absorbance was measured at 450 nm with a microplate reader (GloMax® Discover System).

Statistical analysis

The differences in stem cell marker mRNA expression levels or inhibitor effects between non-CSCs and CSCs were analyzed using the Student’s t-test. The differences in ion transport-related molecule mRNA expression levels between non-CSCs normoxia, non-CSCs hypoxia, and CSCs normoxia were analyzed using the Tukey–Kramer test. The Smirnov-Grubbs’ test was used to evaluate the outliers of ion transport-related molecule mRNA expression levels. Statistical analyses were performed using Statview software for Windows (SAS Institute, Cary, NC, USA).

Results

Obtainment of CSCs using the sphere formation assay

CSCs of U-251 MG and U-87 MG cells were generated using the sphere formation assay, and the formation of spheres was confirmed by an inverted microscope (IX70 Olympus, Tokyo, Japan) (Fig. 1a). The sphere-forming assay is a common technique used to examine the function of cancer stem cells [22, 23]. Tumor cells almost die in the
process of forming spheres (when cultured under non-adherent conditions), and cancer stem cells proliferate. Total RNA was extracted from spheres, and the mRNA levels of stem cell markers \((\text{Nestin, Nanog, CD133, Sox-2, and Oct-4})\) were compared between adherent cells and spheres cultured in 20 \% O\(_2\) by qRT-PCR. \textit{Nestin} was upregulated in U-251 MG cells, and \textit{Nestin} and \textit{CD133} were upregulated in U-87 MG cells (Fig. 1b). Immunofluorescence analysis using a confocal laser microscope showed the upregulation of \textit{Nestin} and \textit{Nanog} in U-251 MG cells, and the upregulation of \textit{Nestin} and \textit{CD133} in U-87 MG cells (Fig. 1c). Therefore, a population of cells containing many stem cells was obtained using the sphere formation assay.

In the following experiments, spheres were considered CSCs and adherent cells were considered non-CSCs. In addition, spheres were treated with pimonidazole, which is reduced under hypoxic conditions and aggregates in cells. The accumulation of pimonidazole in spheres was confirmed by immunofluorescence staining, indicating that intracellular conditions were hypoxic (Fig. 1d).

The mRNA expression levels of ion transport-related molecules in non-CSCs and CSCs

To analyze the changes in the mRNA expression levels of ion transport-related molecules, samples of non-CSCs normoxia, CSCs normoxia, and non-CSCs hypoxia were prepared and subjected to qRT-PCR. The following transport-related molecules were analyzed: CA (CA9, CA12), NHE (NHE1, NHE2), and MCT (MCT1, MCT4). CA9 and CA12 mRNA transcripts were upregulated in HA cells and CSCs in both cell lines (U-251 MG; CA9 in HA cells: \(26.12 \pm 2.34\), \(CA9\) in CSCs: \(38.91 \pm 5.84\), \(CA12\) in HA cells: \(3.30 \pm 1.20\), \(CA12\) in CSCs: \(44.58 \pm 4.95\), U-87 MG; \(CA9\) in HA cells: \(21.47 \pm 2.27\), \(CA9\) in CSCs: \(23.42 \pm 5.20\), \(CA12\) in HA cells: \(1.61 \pm 0.18\), \(CA12\) in CSCs: \(3.61 \pm 0.70\)). In the U-251 MG cells, both \textit{NHE1} and \textit{NHE2} transcripts were downregulated in HA cells and CSCs, whereas \textit{NHE2} transcripts were downregulated in HA cells and CSCs, whereas \textit{NHE2} was upregulated in CSCs in U-87 MG cells (U-251 MG; \textit{NHE1} in HA cells: \(0.66 \pm 0.10\), \textit{NHE1} in CSCs: \(0.22 \pm 0.01\), \textit{NHE2} in HA cells:
Based on these observations, we were focused on MCT1, which were upregulated in CSCs to a greater extent than in HA cells.

**The pHMBS (MCT inhibitor) was more cytotoxic at lower concentrations in CSCs than in non-CSCs in U-251 MG cells and inhibited sphere formation**

To analyze the effects of MCT inhibition, non-CSCs or CSCs of U-251 MG and U-87 MG cells were treated with pHMBS. The IC$_{50}$ was approximately 260 µM in non-

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**Fig. 2** mRNA expression of ion transport-related molecules in non-CSCs normoxia (white bars), non-CSCs hypoxia (gray bars), and CSCs normoxia (black bars). CA9 and CA12 transcripts were upregulated by hypoxia in U251-MG and U87-MG cell lines. CA9, CA12, and MCT1 transcripts were upregulated by CSCs in these cell lines ($n = 3–5$, mean ± SEM; *$p < 0.05$, **$p < 0.01$ vs. non-CSC normoxia. 1$p < 0.05$, 1$p < 0.01$ vs. non-CSC hypoxia). CSC cancer stem cell, CA carbonic anhydrase, NHE Na$^+$–H$^+$ exchanger, MCT monocarboxylate transporter
CSCs and 200 μM in U-251 MG cells, and approximately 150 μM in non-CSCs and 100 μM in CSCs in U-87 MG cells. Our results showed that pHMBS had a lower IC₅₀ in CSCs than in non-CSCs. However, to show the full inhibitory effects, we used higher concentrations of pHMBS in CSCs than in non-CSCs of U-87 MG cells (Fig. 3a). The size of CSC spheres of U251-MG cells as well as U-87 MG cells became smaller by pHMBS treatment (Fig. 3b). We next examined the intracellular amounts of lactic acid using lactate assay. The pHMBS treatment accumulated much more lactic acid in CSCs normoxia than in non-CSCs normoxia. The intracellular lactate density in U251-MG CSCs under normoxic conditions increased to approximately 470 %, whereas that in U251-MG non-CSCs under normoxic conditions increased to approximately 130 %. The intracellular lactate density in U87-MG CSCs under normoxic conditions increased to approximately 170 %, whereas that in U87-MG non-CSCs under normoxic conditions increased to approximately 110 % (Fig. 3c).

Fig. 3 Effect of pHMBS on non-CSCs and CSCs. a Cells were treated with pHMBS (U-251 MG: 200–360 μM, U-87 MG: 100–260 μM) or vehicle for 72 h (n = 3, mean ± SEM; *p < 0.05, **p < 0.01 vs. non-CSC of each concentration). b The number and size of spheres treated for 6 days with various concentrations of pHMBS. Scale bars 300 μm. c The intracellular lactate density (nmol/1.0 × 10⁶ cells) of non-CSCs or CSCs treated with pHMBS for 72 h (non-CSCs) or 6 days (CSCs). These data are representative of two independent experiments. CSC cancer stem cell

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AR-C117977 (MCT1 selective inhibitor) was more cytotoxic at lower concentrations in CSCs than in non-CSCs in each cell line and inhibited sphere formation.

To analyze the effect of selective MCT1 inhibition, non-CSCs or CSCs of U-251 MG and U-87 MG cells were treated with AR-C117977. The IC_{50} was approximately 10 μM in non-CSCs and 2.5 μM in CSCs in U-251 MG cells, and approximately 15 μM in non-CSCs and 10 μM in CSCs in U-87 MG cells. These results indicated that AR-C117977 treatment reduced cell viability at lower concentrations in CSCs than in non-CSCs (Fig. 4a). The maximum diameter of spheres decreased in response to AR-C117977 in a dose-dependent manner (Fig. 4b). In the analysis of the intracellular lactic acid density, AR-C117977 also increased the intracellular lactic acid contents, and more lactic acid was accumulated in CSCs normoxia than in non-CSCs normoxia. The intracellular lactate density in U251-MG CSCs under normoxic conditions increased to approximately 240 %, whereas that in U251-MG non-CSCs under normoxic conditions increased 300 %.

Fig. 4 Effects of AR-C117977 on non-CSCs and CSCs. a Cells were treated with AR-C117977 (0.5–25 μM) or 0.2 % DMSO (vehicle) for 72 h (n = 3, mean ± SEM; *p < 0.05, **p < 0.01 vs. non-CSC of each concentration). b The number and size of spheres treated for 6 days with various concentrations of AR-C117977. Scale bars 300 μm. c The intracellular lactate density (nmol/1.0 × 10^4 cells) of non-CSCs or CSCs treated with AR-C117977 for 72 h (non-CSC) or 6 days (CSC). These data are representative of two independent experiments. CSC cancer stem cell.
to approximately 130%. The intracellular lactate density in U87-MG CSCs under normoxic conditions increased to approximately 120%, whereas that in U87-MG non-CSCs under normoxic conditions increased to approximately 105% (Fig. 4c). These observations suggest that MCT1 plays a more important role in the maintenance of homeostasis in GSCs.

Discussion

The present study indicates that the HA cells or CSCs from glioblastoma cells induced changes in the expression levels of various ion transport-related molecules. In particular, the conversion of cancer cells to CSCs induced the upregulation of CA9, CA12, and MCT1, whereas the conversion to HA cells induced CA9 upregulation in U-251 MG and U-87 MG cells, as reported previously [24]. Immunofluorescence analysis showed the accumulation of pimonidazole in spheres, which suggested that intracellular conditions were hypoxic (Fig. 1d). The increased expression level of CA9 in CSCs was induced by the hypoxic environment of spheres. In addition, the CSCs hypoxia could not induce the upregulation of CA9 by so much. However, CA9 was further upregulated in CSCs hypoxia (Fig. S1). These results suggested that MCT1, which was upregulated only during the conversion of non-CSCs to CSCs, plays an important role in the maintenance of CSCs (Fig. S2). In this study, we used b-FGF and EGF to produce CSC spheres. This is a common technique used to examine the function of cancer stem cells [22, 23]. However, the detailed mechanism of how these cytokines act on MCT1 expression has not been clarified. Suzuki et al. showed that b-FGF and EGF promote the uptake of Glutamate by upregulating the glial glutamate transporter (GLAST) [25]. Therefore, we speculate that b-FGF and EGF signaling might also upregulate MCT1 expression. Consequently, glioblastoma stem cells (GSCs) have higher sensitivity to MCT1 inhibition as shown below. However, the accurate mechanisms have yet to be clarified. A previous study identified 14 MCT isoforms, including the three isoforms MCT1, MCT2, and MCT4, which are expressed in the brain [26]. MCT1 is expressed in astrocytes, endothelial cells of capillaries, and oligodendrocytes; MCT4 is primarily expressed in astrocytes, whereas MCT2 is expressed in neurons [26]. In addition, MCT1 and MCT4 control the release of lactic acid from astrocytes, and MCT2 controls the uptake of lactic acid into neurons [26].

We first examined the inhibiting effect of pHMBS, which inhibits MCT1 and MCT4 by binding to their cofactor CD147 (basigin) [27–30]. The observations that the size of CSC spheres of each cell lines became smaller by pHMBS treatment suggests that this inhibitor caused cell death because the cells are unable to maintain homeostasis [31, 32], which is in turn associated with the inhibition of the release of lactic acid. In CSCs normoxia, pHMBS decreased the sphere-forming ability and induced cell death earlier than non-CSCs normoxia. However, a higher concentration of pHMBS was needed to completely induce CSC death in U-87 MG cells than in non-CSCs. The concentration of pHMBS required to induce cell death in U-251 MG cells (approximately 200 μM) was higher than that required to induce cell death in U-87 MG cells (approximately 100 μM). We think that this is because the expression levels of MCT1 and MCT4 in U-87 MG cells were low compared with those in U-251 MG cells (Fig. S1). These data indicate that the dispersion effect depends on the type of glioblastoma.

We next examined the effect of selectively inhibiting MCT1 by AR-C117977, which was recently developed as an immunosuppressant and selectively binds to and inhibits MCT1 [33, 34]. In CSCs, AR-C117977 inhibited the sphere-forming ability, and cell death was induced earlier than in non-CSCs at all concentrations. These results showed an inverse correlation to the expression level of MCT1: higher MCT1 expressing cells showed cell death at lower concentrations by AR-C117977. For the expression in the plasma membrane, MCTs require binding partners: CD147 (basigin) and gp70 (embigin). MCT1 associates with gp70 (embigin) in spite of the absence of CD147 [35]. pHMBS inhibits both MCT1 and MCT4 by binding to CD147 (basigin), and therefore pHMBS cannot inhibit MCT1 sufficiently. In contrast, AR-C117977 directly inhibits MCT1 [34]. Taken together, we speculate that a specific MCT1 inhibitor AR-C117977 is more effective in cells maintaining CSC homeostasis through MCT1. Furthermore, MCT1 inhibition induces the accumulation of protons in the cytoplasm, resulting in the acidification of GSCs. Because the accumulation of protons as well as Cl ions, which are counter ions of protons, is associated with cellular functions including migration and autophagy [36–38], the intracellular acidification might also induce cell death. We think that selective inhibition of MCT1 can decrease side effects in therapy because MCTs express the various organs not only brain and tumor [26]. pHMBS and AR-C117977 were used in animal studies in several reports, and no significant side effects were reported [27, 28, 30, 33]. However, when considering the clinical settings, pHMBS might be inappropriate because it contains mercury. Moreover, a phase I clinical trial of AZD3965, a MCT1 selective inhibitor, has started in patients with advanced cancer (NCT01791595). Therefore, we think that MCT1 selective inhibitors are safe in a clinical setting.

In conclusion, the selective inhibition of MCT1 demonstrated cytotoxicity at low concentrations in glioblastoma cells against inhibition of MCT and was
highly effective against glioblastoma-derived CSCs. These findings suggest that MCT1 selective inhibitors are useful as targeted therapeutic agents against GSCs.

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