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RESEARCH ARTICLE

Identification of 2-Fluoropalmitic Acid as a Potential Therapeutic Agent Against Glioblastoma

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ARTICLE HISTORY

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Abstract: ***Background*:** Glioblastomas (GBMs) are aggressive malignant brain tumors. Although chemotherapy with temozolomide (TMZ) can extend patient survival, most patients eventually demonstrate resistance. Therefore, novel therapeutic agents that overcome TMZ chemoresistance are required to improve patient outcomes.

***Purpose*:** Drug screening is an efficient method to find new therapeutic agents from existing drugs. In this study, we explored a novel anti-glioma agent by drug screening and analyzed its function with respect to GBM treatment for future clinical applications.

***Methods*:** Drug libraries containing 1,301 diverse chemical compounds were screened against two glioma stem cell (GSC) lines for drug candidate selection. The effect of selected agents on GSCs and glioma was estimated through viability, proliferation, sphere formation, and invasion assays. Combination therapy was performed to assess its ability to enhance TMZ cytotoxicity against GBM. To clarify the mechanism of action, we performed methylation-specific polymerase chain reaction, gelatin zymography, and western blot analysis.

***Results*:** The acyl-CoA synthetase inhibitor 2-fluoropalmitic acid (2-FPA) was selected as a candidate anti-glioma agent. 2-FPA suppressed the viability and stem-like phenotype of GSCs. It also inhibited proliferation and invasion of glioma cell lines. Combination therapy of 2-FPA with TMZ synergistically enhanced the efficacy of TMZ. 2-FPA suppressed the expression of phosphor-ERK, CD133, and SOX-2; reduced MMP-2 activity; and increased methylation of the *MGMT* promoter.

***Conclusion*:** 2-FPA was identified as a potential therapeutic agent against GBM. To extend these findings, physiological studies are required to examine the efficacy of 2-FPA against GBM *in vivo*.

Keywords: Glioma, Drug screening, 2-fluoropalmitic acid, Temozolomide, Matrix metalloproteinase, Glioma stem cells.

1. INTRODUCTION

Glioblastoma (GBM) is an aggressive type of brain cancer and remains incurable despite major advances in the diagnosis and treatment. The prognosis of most patients with GBM remains poor, with a median overall survival of <15 months when treated with temozolomide (TMZ) chemotherapy, radiotherapy, and surgical resection [1-3]. As the gold standard chemotherapeutic for GBM, TMZ has increased survival and improved quality of life [4, 5]. However, TMZ resistance is the primary reason for poor prognosis, possibly owing to the glioma cancer stem cell (GSC) population [6, 7]. Strategies to overcome TMZ resistance and increase efficacy are now being investigated.

Inherent issues with drug development, including high cost and high risk of failure, have necessitated the use of alternative methods for drug development. As an efficient method to identify new potential anti-glioma agents, high-throughput screening (HTS) of existing drugs provides promising options to overcome issues associated with typical drug development [8, 9]. In the past, several drugs were identified using HTS, including the anti-schizophrenia drug fluspirilene [9] and the glycogen synthase kinase 3β inhibitor kenpaullone as potential glioma therapeutic agents [10].

In this study, we performed HTS using GSCs and cell viability assays for testing an existing drug library, which contains 1,301 diverse chemical compounds (Supplementary Fig. **1**), as shown in our previous studies [9-11]. We identified 2-fluoropalmitic acid (2-FPA), a fatty acid inhibitor that inhibits sphingosine biosynthesis and long-chain acyl-CoA synthetase (ACSL) [12], as a novel therapeutic agent for GBM. We found that the antitumor effect of this agent involved attenuating the activity of extracellular signal-regulated kinase (ERK), an essential molecule involved in many biological processes such as proliferation [1] and involved suppressing GSC stemness. We also found that 2-FPA enhanced TMZ efficacy against glioma as a combination therapy.

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2. MATERIALS AND METHODS

2.1. Drug Screening

In this study, we performed high-throughput screening (HTS) using glioma stem cell line (GSC) by cell viability assay with existing drug libraries, which contain 1,301 diverse chemical compounds [FDA Approved Drug Library (640), ICCB Known Bioactive Library (480), Kinase Inhibitor Library (80), Fatty acid Drug Library (68), Phosphatase Inhibitor Library (33)], as shown in previous studies [9-11]. HTS is composed of three steps: high concentration (1, 5, and 20 μM) WST-8 assay to exclude ineffective candidates, excluding reported drugs in PubMed, and lower concentration (0.1, 0.5, and 1 μM) WST-8 assay to exclude ineffective agents. Candidate drug was identified as demonstrating remarkable inhibition of cell viability at a higher concentration, as well as express the ability to enhance the efficacy of TMZ against glioma treatment at a lower concentration (Supplementary Fig. **1**).

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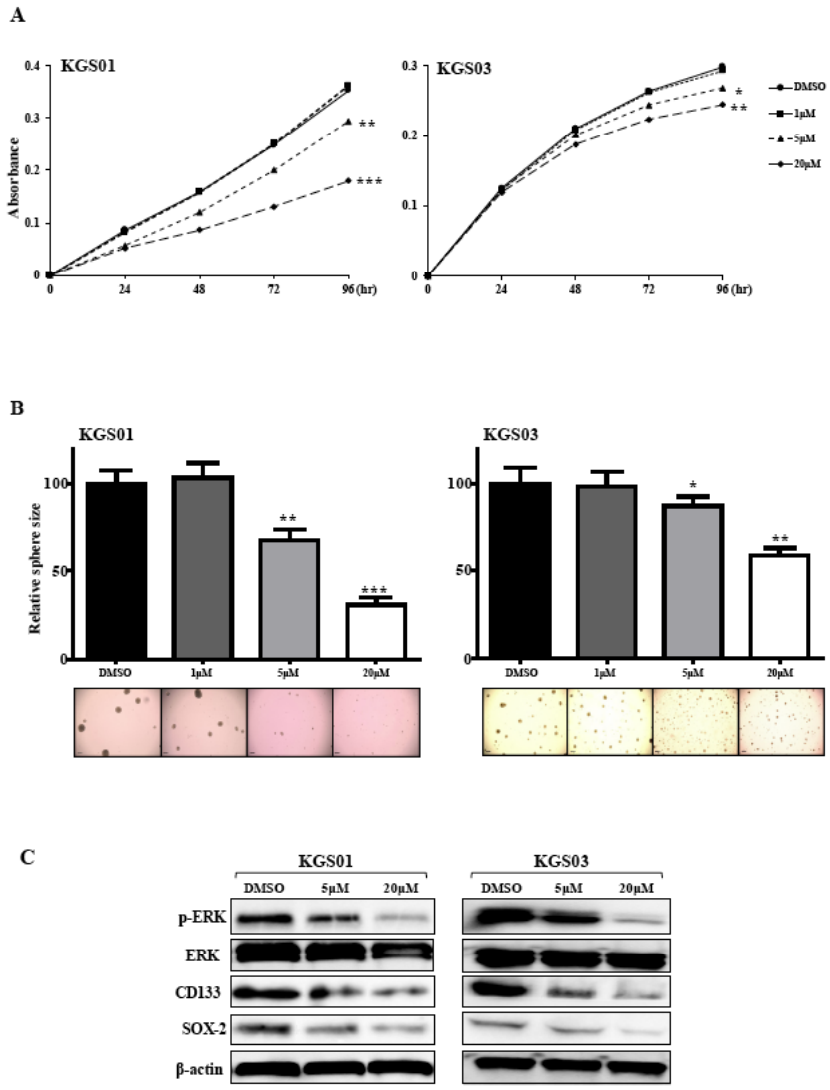


Fig. (1). Effect of 2-fluoropalmitic acid (2-FPA) on glioma stem cells (GSCs), stem cell markers, and p-ERK expression.
(A) GSCs were seeded in 96-well plates and treated with vehicle (DMSO) or 1, 5, or 20 μM of 2-FPA. CCK-8 reagent was added after 24, 48, 72, and 96 h of incubation. After a 4-h incubation with CCK-8, the plate was read on a microplate reader. Each experiment was repeated at least three times. Results are shown as the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus DMSO. (B) Sphere-forming assay was performed using the GSC lines KGS01 and KGS03. The sphere numbers were counted 7 days after treatment with vehicle (DMSO) or 1, 5, or 20 μM of 2-FPA. Each experiment was repeated at least three times. 2-FPA at 5 and 20 μM suppressed sphere formation compared with DMSO. Results are shown as the mean ± SD of three independent experiments. Bars = 200 μm; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus DMSO. (C) GSCs were grown in the presence of different concentrations of 2-FPA for 24 h at 37 ℃, followed by cell lysis and protein extraction. The effects of 2-FPA on ERK signaling, as well as the stem cell markers CD133 and SOX2, were investigated by western blotting. The expression of p-ERK, CD133, and SOX-2 was suppressed by 5 and 20 μM of 2-FPA in both GSC lines. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

2.2. Cell Culture

Human patient-derived GSC lines KGS01 and KGS03 were established at Kanazawa University. All materials and protocols were approved by the Ethics Committees of Kanazawa University. Both cell lines have been confirmed to comprise tumor-initiating cells in previous studies [9, 10]. GSCs were cultured in neurosphere formation medium containing Dulbecco's Modified Eagle's Medium (DMEM/F12; Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with recombinant human epidermal growth factor at 20 ng/mL (Sigma–Aldrich, St. Louis, MO, USA), recombinant human basic fibroblast growth factor at 20 ng/mL (Sigma–Aldrich), MACS® NeuroBrew-21 supplement without vitamin A (Miltenyi

Biotec, North Rhine-Westphalia, Germany), GlutaMAX (Gibco), and 1% penicillin/streptomycin (Gibco).

Human GBM cell lines, A172, U87, and U251, were purchased from the European Collection of Authenticated Cell Culture (ECACC) in November 2014. Cell lines were characterized at the Resource Institute by short tandem repeat profile analysis. Contamination was tested by mycoplasma detection kit (InvivoGen, San Diego, CA, USA). Authentication of cell lines was unnecessary because they were expanded by culturing them for fewer than two passages and stored at -80°C. Cells were cultured at 37 °C in a 5% CO_2 incubator and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma–Aldrich).

2.3. Cell Viability Assay

GSC viability was assessed using the Cell Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) following the manufacturer's instructions. Briefly, GSC spheres were dissociated into single cells using StemPro Accutase (Gibco). Then, cells were seeded into a 96-well Costar ultra-low attachment plate (Corning Inc, New York, USA) at a density of 1×10^3 cells/100 µL and treated with 1, 5, or 20-µM of 2-FPA or 100-µM TMZ. The relative numbers of viable cells were determined by measuring the absorbance at 0, 24, 48, 72, and 96 h with the CCK-8 reagent using an iMARK^{TM}microplate reader (BIO-RAD, Hercules, CA, USA).

The Alamar blue assay (Biosource, Camarillo, CA, USA) was performed to examine GBM cell proliferation. Briefly, 1×10^3 GBM cells were seeded into a 96-well plate in 200 µL of culture medium supplemented with 0.1% FBS. After a 4-h incubation at 37 °C, 20 µL of Alamar blue was added to each well. The plate was read using a fluorescence plate reader at 0, 24, 48, 72, and 96 h. Average fluorescence values from eight wells were calculated and plotted. To investigate the effect of 2-FPA on proliferation, cells were treated with various concentrations (1, 5, or 20 µM) of 2-FPA (Cayman Chemical Company, Ann Arbor, MI, USA). To check whether the combination of 2-FPA with TMZ was more effective than either agent alone, cells were treated with 100-µM TMZ (Sigma–Aldrich) alone or combined with 1-µM 2-FPA. Dimethyl sulfoxide (DMSO), the organic solvent of 2-FPA, was used as a negative control.

2.4. Tumor Sphere-Forming Assay

The sphere-forming assay was performed as described previously [13, 14]. Briefly, KGS01 and KGS03 GSC spheres were dissociated into single cells with StemPro Accutase (Gibco). Then, 3×10^3 single cells were seeded in a 96-well Costar ultra-low attachment plate (Corning) in 200 µL of neurosphere medium supplemented with 1.0% methylcellulose. Cells were treated with different concentrations of 2-FPA, 100-µM TMZ, or DMSO. After 7 days of incubation, the tumorspheres with diameters ≥ 50 µm were measured using Image J software, and their average was calculated.

2.5. Western Blot Analysis

Cells were treated with different concentrations of 2-FPA and collected. RIPA buffer (FUJIFILM, Tokyo, Japan) containing 1% protease and phosphatase inhibitors (Sigma–Aldrich) was used for the preparation of cellular lysates. Total protein was extracted from cultured cells, and protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Primary antibodies against the following markers were used: p-ERK1/2 (T202/Y204), ERK1/2 (#4370, #4695, Cell Signaling Technology Beverly, MA, USA), CD133 (#36131, Bioworld Technology, Inc, MN, USA), SOX-2 (#42620, GeneTex, CA, USA), ACSL5 (#1716, Santa Cruz Biotechnology, Inc, TX, USA), p-KAP1S824, KAP1, p-Histone H2AX S139 (ab70369, ab109545, ab81299, Abcam, Cambridge, MA, USA), and β-actin (#013-24553, Wako, Osaka, JAPAN), as described previously [15]. After secondary antibody incubation, membranes were exposed to Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific) for 5 min, and bands were detected using a Light Capture II cooled CCD Camera System.

2.6. Methylation-Specific Polymerase Chain Reaction (PCR) Assay

The effects of 2-FPA on the methylation status of the *MGMT* promoter were evaluated by methylation-specific PCR assays as described previously [16]. A172 and U251 cells were serum-starved and treated with 1-µM 2-FPA for 72 h. Genomic DNA was extracted from cells using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Sodium bisulfite conversion of 1 µg of purified DNA was performed using the EpiTect Bisulfite Kit (QIAGEN) according to the manufacturer's protocol. Methylation-specific PCR of bisulfate-converted DNA was carried out *via* a nested, two-stage PCR approach as described previously [17] using the GeneAmp PCR System 2700 (Applied Biosystems, Grand Island, NY, USA). U87 and U138 cell lines were used as methylated and unmethylated controls, respectively. Amplified PCR products were separated by 3% agarose gel electrophoresis and visualized with ethidium bromide staining.

2.7. Matrigel Invasion Assay

Cell invasion was assessed using modified Boyden chambers consisting of Transwells with Matrigel-pre-coated membrane filter inserts in 24-well tissue culture plates (BD Biosciences, San Jose, CA, USA), as previously described [15]. Serum-starved GBM cells were treated with different concentrations of 2-FPA, suspended in DMEM supplemented with 0.1% FBS, and added to Transwells at 5×10^4 cells/500 µL. Then, Transwells were immersed in DMEM supplemented with 10% FBS. After 12–20 h of incubation at 37 °C, non-invasive cells were removed by wiping the upper side of the filter, and the invasive cells were fixed with methanol and stained using a Diff-Quik kit (Sysmex, Kobe, Japan). Nine microscopic fields were randomly selected on the lower side of the filter, and the invaded cells were counted. The mean number of invasive cells was calculated and analyzed.

2.8. Gelatin Zymography Assay

GBM cells were serum-starved and treated with different concentrations of 2-FPA at 37 °C for 24 h, and the release of matrix metalloproteinase 2 (MMP-2) was assessed using gelatin zymography as described previously [18]. Briefly, samples were prepared by adding an equivalent amount of tris-glycine SDS sample buffer (2×) (Thermo Fisher Scientific, MA, USA) to the culture medium and incubating for at least 5 min at 22 ± 5 ℃; separation was conducted using 10% Zymogram Plus Gelatin Gel Electrophoresis (Thermo Fisher Scientific). Next, gels were washed in renaturing buffer containing 2.5% (v/v) triton X-100 at 22 ± 5 ℃ and incubated in developing buffer containing 10-mM $CaCl_2$, 1% NaN_3, and 40-mM tris-HCl (pH 8.0) at 37 ℃ by shaking overnight. Finally, MMP gels were rinsed with distilled water and stained for 30 min with SimplyBlue^{TM} SafeStain (Thermo Fisher Scientific). The gelatinolytic activities were densitometrically quantified using ImageJ and analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., CA, USA).

2.9. Statistical Analysis

The data are presented as the mean ± standard deviation (SD). Statistically significant differences in the mean or median values between the two groups were tested using the Student's *t*-test or Mann–Whitney *U*-test as appropriate. P values less than 0.05 were considered statistically significant differences. All statistical calculations were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., CA, USA).

3. RESULTS

3.1. Effect of 2-FPA on GSC Viability and Stem Cell Properties

To determine the effect of 2-FPA on GSCs, cell viability was evaluated using the CCK-8 cell viability assay. As shown in Fig. 1A, 2-FPA significantly suppressed the viability of GSCs at 5 and 20 μM in a dose-dependent manner, but 1-μM 2-FPA did not significantly suppress GSC viability. Because 2-FPA suppressed GSC viability, we examined whether 2-FPA can influence the stem cell properties of GSCs by performing sphere-forming assays and evaluating stem cell markers after 2-FPA treatment. 2-FPA decreased the sphere-forming ability of both GSC cell lines in a dose-dependent manner, with significantly decreased sphere sizes after treatment with 5-μM and 20-μM 2-FPA (Fig. **1B**). However, 1-μM 2-FPA did not have any effect on sphere formation, which is consistent with the results of the cell viability assay. We next examined the expression of stem cell markers CD133 and SRY (sex-determining region Y)-box (SOX) 2. We found that 2-FPA attenuated the expression of CD133 and SOX2 in a dose-dependent manner in both GSC cell lines (Fig. 1C). These data indicate that the reduced sphere-forming ability of GSCs was associated with reduced CD133 and SOX2 expression induced by 2-FPA.

To determine the potential mechanism through which 2-FPA suppresses cell viability and stemness, we analyzed various signal transduction pathways, including the ERK pathway, protein kinase B (also known as AKT) (Supplementary Fig. 2), and mitogen-activated protein kinase (MEK) pathways. 2-FPA reduced the expression level of p-ERK at 5 μM and 20 μM in both GSC lines (Fig. 1C). Of all proteins tested, ERK was the only protein significantly altered by 2-FPA treatment.

3.2. Effect of 2-FPA on the Proliferation of GBM Cells Via the Inhibition of p-ERK

Three glioma cell lines (A172, U251, and U87) were used to assess the effects of 2-FPA on proliferation. 2-FPA significantly suppressed the proliferation of all three lines in a dose-dependent manner, although no significant effects were observed with 1-μM 2-FPA (Fig. **2A**). We also analyzed various signal transduction pathways that are important regulators of tumor cell proliferation. Western blot analysis of glioma cell lines showed similar results with those obtained using GSCs. 2-FPA suppressed p-ERK in all glioma lines (Fig. **2B**). However, 1-μM 2-FPA did not decrease p-ERK, consistent with our earlier results.

3.3. Effect of 2-FPA on TMZ Efficacy Against GSCs

To identify the effect of 2-FPA on TMZ efficacy, we treated GSCs with 2-FPA, TMZ, and a combination of 1-μM 2-FPA and 100-μM TMZ. Our results showed that monotherapy with either 2-FPA or TMZ did not reduce cell viability. However, combination treatment significantly inhibited the viability of both GSC cell lines (Fig. **3A**) and significantly decreased the sphere formation of both GSC lines compared with DMSO, 2-FPA, and TMZ treatment alone (Fig. **3B**). Sphere sizes were also reduced in both GSC lines treated with TMZ combined with l-μM of 2-FPA. These data imply that 2-FPA enhances the cytotoxic effect of TMZ toward GSCs.

3.4. Influence of 2-FPA on the Effects of TMZ Against GBM Cells by Enhancing *MGMT* Promoter Methylation

To identify how 2-FPA might influence the efficacy of TMZ against GBM cell lines, we treated A172, U251, and U87 cells with DMSO, 2-FPA, TMZ, or combination of low-dose 2-FPA and TMZ. Our results showed that single treatment with 2-FPA did not inhibit cell proliferation. However, combination treatment significantly inhibited the proliferation of all three cell lines, consistent with our previous results (Fig. **4A**).

To investigate the mechanism through which combination therapy suppressed proliferation, we evaluated the effect of 2-FPA treatment on *MGMT* promoter methylation. 2-FPA treatment decreased levels of the unmethylated promoter in A172 and U251 cell lines but did not alter the *MGMT* promoter methylation status of U87 cells (which is always methylated and used as a positive control for methylation) (Fig. **4B**).

3.5. Effect of 2-FPA on the Invasion of GBM Cells by Inhibiting MMP-2 Activity

We next evaluated the effect of 2-FPA on glioma invasion. We found that 2-FPA inhibited the invasion of GBM cells to the extracellular matrix in a dose-dependent manner (Fig. 5A). Unlike previous results, the invasion ability of all glioma cell lines was inhibited with all doses of 2-FPA, including 1 μM. Numbers of invaded GBM cells were reduced by 32.3% and 71.2% in A172 ($p < 0.01$), 31.4% and 67.4% in U251 ($p < 0.01$), and 16.7% and 65.8% in U251 ($p < 0.05$) cells with 1-μM and 8-μM 2-FPA, respectively.

To investigate the mechanism through which 2-FPA inhibits glioma cell invasion, we evaluated the effect of 2-FPA on MMP-2 by gelatin zymography. Densitometry analysis of gelatin zymography gels indicated that 2-FPA suppressed the secretion of MMP-2 in a dose-dependent manner. In all three cell lines, the pro-MMP-2 expression did not change but active MMP-2 decreased after 2-FPA treatment (Fig. 5B). The ratio of active MMP-2 to pro-MMP-2 decreased in a dose-dependent fashion in 2-FPA groups compared with that in the DMSO group. Taken together, 2-FPA suppressed the invasion of glioma cell lines *via* attenuating the secretion of active MMP-2.

4. DISCUSSION

In this study, we identified 2-FPA as a novel potential treatment agent for glioma. 2-FPA was previously identified as a comprehensive inhibitor of ACSLs [12]. ACSLs are enzymes that activate long-chain fatty acids by catalyzing the formation of fatty acyl-CoA [19] and function in the β-oxidation of fatty acids and the lipid modification of cellular proteins [20]. According to previous studies, ACSLs are overexpressed in many cancers including glioma [21], and their inhibition results in selective cytotoxicity toward malignant cancer cells [22]. All these observations indicate that ACSLs act as cancer survival factors [23]. Among them, the ACSL5 isozyme is frequently overexpressed in human glioma and promotes glioma cell survival under extracellular acidic conditions [24]. However, in this study, 2-FPA did not change the expression of ACSL5 (Supplementary Fig. **3**). 2-FPA does not affect ACSL5 expression but probably influences its kinase activity at other positions or *via* other unknown pathways. In a previous study, fluorine-substituted palmitic acid (FPA) was found to inhibit ACSL activity [12]. Radiolabeled palmitic acid and FPA were found to decrease the formation of labeled palmitoyl-CoA *in vitro*. The other two groups of labeled fatty acids, labeled oleic acid and labeled stearic acids, showed similar results, suggesting a general inhibitory effect of FPA on ACSL activity. However, how FPA inhibits ACSL activity was not mentioned. Our results also suggest that 2-FPA does not affect the expression of ACSL5. One possible reason is that 2-FPA blocks cellular lipid biosynthesis, which influences the normal biosynthetic processes associated with cell membranes such that the cell membrane becomes unstable and weak and cannot resist external stimuli. Another possible reason according to a previous study is that 2-FPA blocks some fatty-acyl-CoA-specific activity to reduce fatty-acyl-CoA-derivatives that are essential for cell survival [12, 20].

2-FPA suppressed the cell viability and phenotype of GSCs in a dose-dependent manner. We also found that 2-FPA suppressed ERK phosphorylation and decreased the expression of stem cell markers CD133 [25, 26] and SOX-2 [27] in a dose-dependent manner in GSCs. ERK plays important roles in maintaining cancer stemness and chemo-immune-resistance in several cancers [28-30].

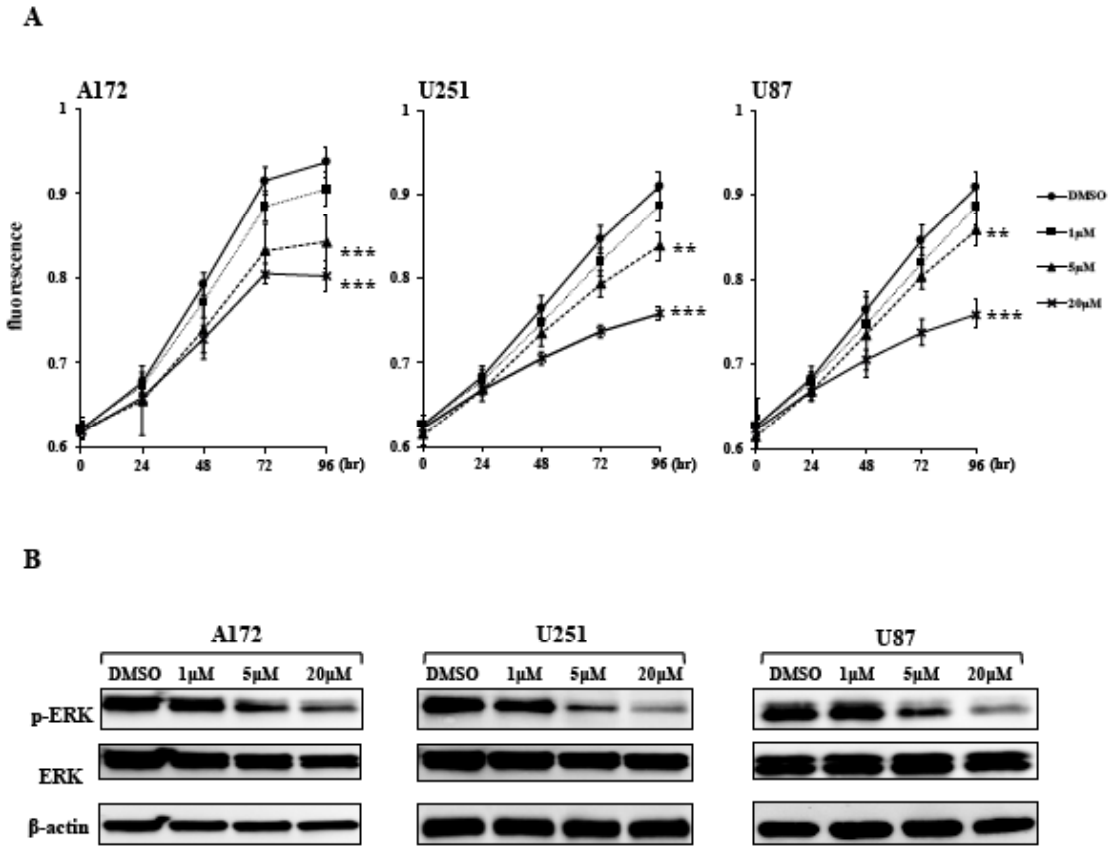
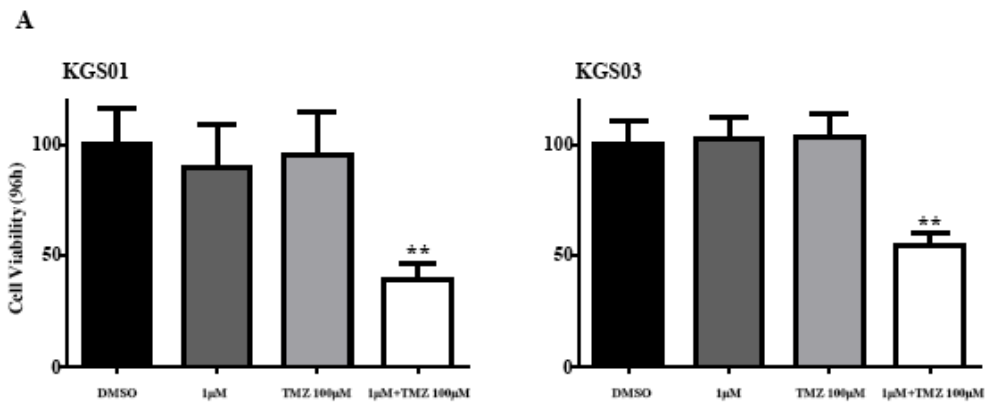


Fig. (2). Effect of 2-fluoropalmitic acid (2-FPA) on glioma cell proliferation and p-ERK.
(A) Three different glioma cell lines were seeded in 96-well plates and grown for 4 h at 37 ℃, followed by the addition of Alamar blue. Cells were treated with DMSO or 1, 5, or 20 μM of 2-FPA. The plate was read on a microplate reader at 24, 48, 72, and 96 h. Each experiment was repeated at least three times. Results are shown as the mean ± SD of three independent experiments. ***, $P < 0.001$ versus DMSO. (B) Cells were grown in the presence of 2-FPA for 24 h at 37 ℃, followed by cell lysis and protein extraction. The effect of 2-FPA on ERK signaling was investigated by western blotting. p-ERK expression was suppressed by 2-FPA in all cell lines. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).



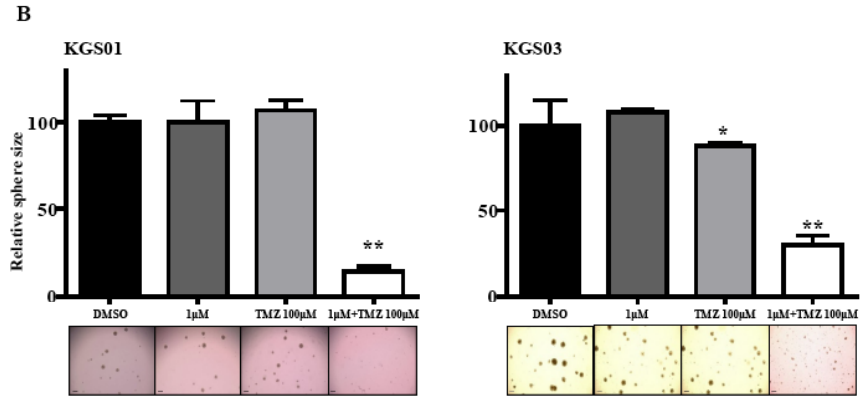


Fig. (3). Effect of 2-fluoropalmitic acid (2-FPA) and temozolomide (TMZ) against glioma stem cells (GSCs).
(A) CCK-8 assays were performed after GSCs were treated with DMSO, 2-FPA, TMZ, or a combination of 2-FPA with TMZ. After 96 h incubation, the absorbance was read on a microplate reader. Each experiment was repeated at least three times. Combination therapy was proven to be more effective at inhibiting GSC viability than either 2-FPA or TMZ alone. Results are shown as mean ± SD of three independent experiments; **, $P < 0.01$ versus DMSO. (B) The sphere-forming assay was performed using the GSC lines KGS01 and KGS03. The sphere numbers were counted 7 days after treatment with DMSO, 2-FPA, TMZ alone, and a combination of 2-FPA with TMZ. Each experiment was repeated at least three times. Combination therapy strongly suppressed sphere formation compared with 2-FPA or TMZ monotherapy. Results are shown as the mean ± SD of three independent experiments. Bars = 200 μm; *, $P < 0.05$; **, $P < 0.01$ versus DMSO. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

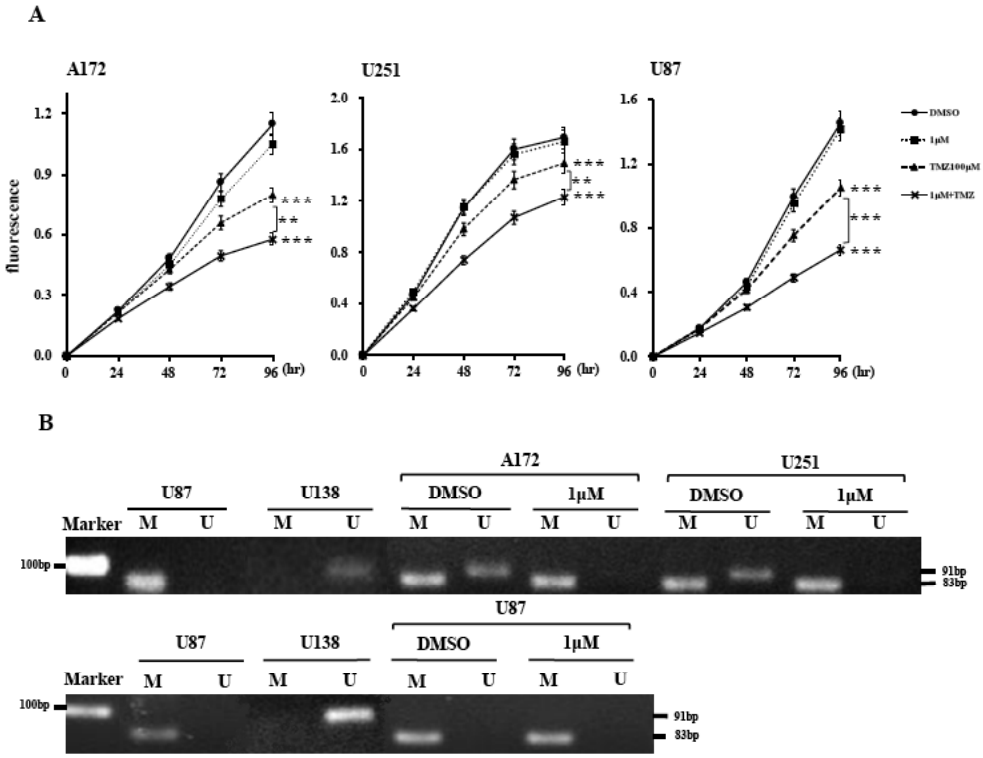


Fig. (4). Effect of 2-fluoropalmitic acid (2-FPA) on temozolomide (TMZ) activity against glioma cell proliferation and *MGMT* promoter methylation.
(A) The Alamar blue assay was performed after glioma cells were treated with either 2-FPA, TMZ, or a combination of 2-FPA with TMZ. The fluorescence was read on a microplate reader at 24, 48, 72, and 96 h. Each experiment was repeated at least three times. Combination therapy was proven to be more effective at inhibiting glioma cell proliferation than either 2-FPA or TMZ alone. Results are shown as the mean ± SD of three independent experiments; **, $P < 0.01$; ***, $P < 0.001$ versus DMSO. (B) 2-FPA treatment decreased methylation of the *MGMT* promoter in A172 and U251 cell lines, whereas the *MGMT* promoter methylation status of U87 was not altered by 2-FPA treatment. U87 and U138 cells were used as positive controls of methylation and unmethylation, respectively. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

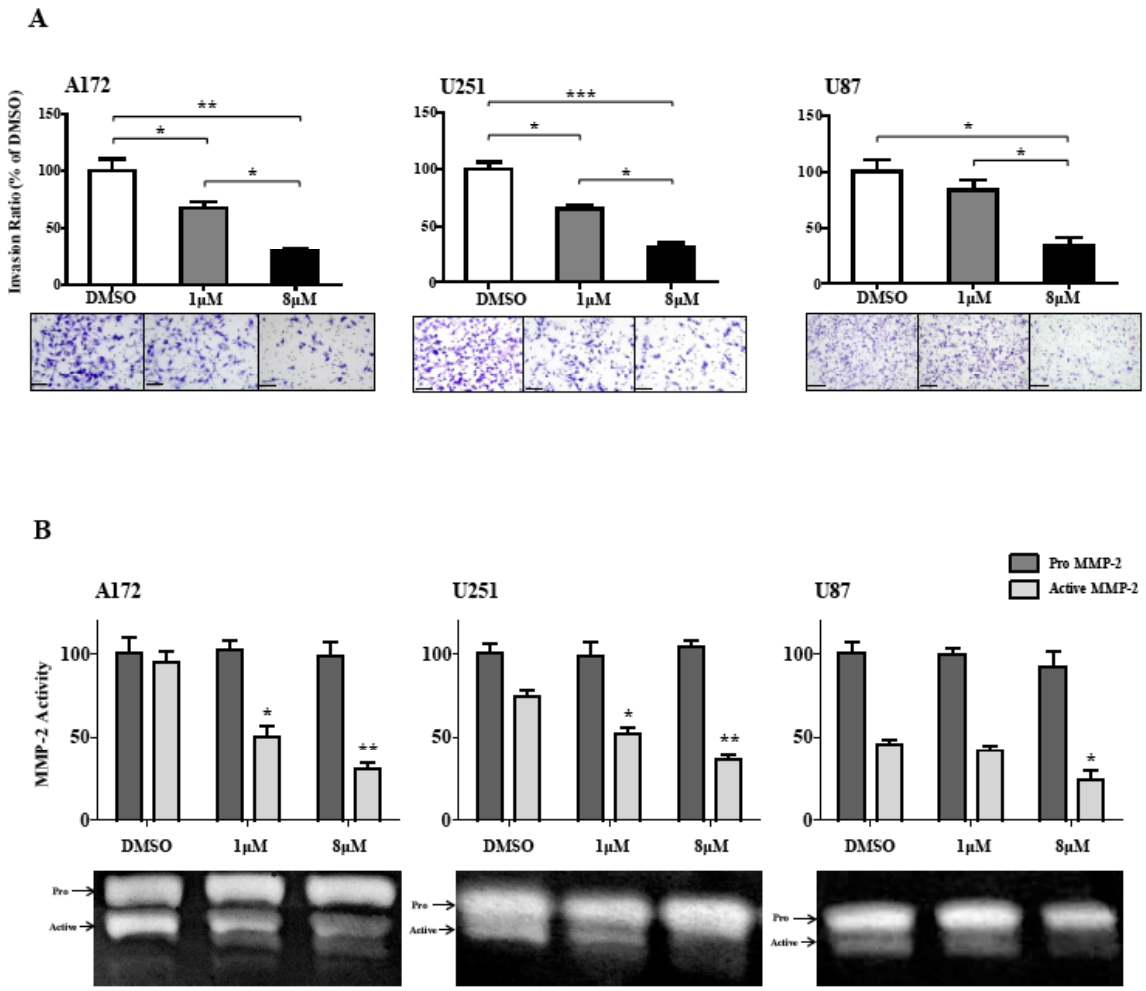


Fig. (5). Effect of 2-fluoropalmitic acid (2-FPA) on glioma cell invasion and MMP-2 activity.
(A) Cells were counted and seeded in the upper wells of Boyden chambers, and then treated with different concentrations of 2-FPA at 37 °C for 12–16 h. Cells that invaded into the lower surface of the membrane were fixed and stained. Mean cell counts from nine different random fields are shown. Bars = 100 μm, mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus DMSO. (B) Cells were grown in the presence of 2-FPA for 24 h at 37 °C, and then the extracellular medium was analyzed by gelatin zymography. MMP-2 activity was indicated through density analysis. 2-FPA inhibited MMP-2 activation in a dose-dependent manner in all cell lines. *, $P < 0.05$; **, $P < 0.01$ versus DMSO. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

We also investigated the effect of 2-FPA on the activity of TMZ against GSCs. A combination of low-dose 2-FPA with TMZ greatly suppressed cell viability and sphere formation in both GSC lines. TMZ resistance is related to MGMT expression levels, which directly correlates with the methylation status of the promoter site in the cytosine-phosphate-guanine *MGMT* gene island [31, 32]. However, the *MGMT* promoter of both GSC lines is known to be methylated (Supplementary Fig. 4); thus, 2-FPA most likely enhances the efficacy of TMZ against GSCs via other pathways. Numerous studies have shown that ERK is essential for many processes including proliferation, the maintenance of stemness, and cell death [29, 33]. In this study, 2-FPA suppressed ERK phosphorylation, which would induce further inhibition of the key physiological processes in GSC development, resulting in the suppression of the GSC phenotype.

Similar to results from the GSC lines, 2-FPA suppressed the proliferation and invasion of glioma cell lines in a dose-dependent manner. We also evaluated molecules that reduce proliferation and found that 2-FPA induced ERK inactivation. ERK is upregulated in glioma cells and is essential for glioma cell proliferation and invasion [34-36]. Studies show that ERK also plays important roles in tumor cell invasion, and ERK inhibition decreases invasion via the inactivation of MMP-2, a Zn-dependent endopeptidase that degrades many extracellular matrix proteins [37-43]. On the basis of these findings, we assessed the effect of 2-FPA on MMP activity. We found that 2-FPA treatment reduced MMP-2 activity in all glioma cell lines in a dose-dependent manner. Taken together, the

suppression of ERK by 2-FPA treatment might attenuate the invasion ability of glioma cells via the inactivation of MMP-2 activity.

Combination treatment of 2-FPA with TMZ synergistically enhanced the efficacy of TMZ against glioma *in vitro*. As the unmethylation of the *MGMT* promoter is the main reason for TMZ resistance [44, 45], we investigated if the methylation status of this promoter was altered by 2-FPA treatment. The results showed that in A172 and U251 lines, the unmethylated status of the *MGMT* promoter was suppressed after treating the cells with 2-FPA. In U87 cells, methylation status was not altered. To confirm the effect of 2-FPA on MGMT, we checked the expression of the MGMT protein, as well as *MGMT* mRNA levels, after 2-FPA treatment (Supplementary Fig. **5**). Both western blot and qPCR analysis showed decreased MGMT expression, which supported the results of methylation-specific PCR. To this end, we also investigated other possible mechanisms, besides *MGMT* promoter methylation. Some studies suggest that TMZ induces DNA methylation in tumor cells causing DNA damage, tumor cell senescence, and G2/M arrest [46]. We checked the expression of KAP1 and the DNA damage repair marker γH2AX to confirm that 2-FPA damaged tumor cell double-strand DNA chains to enhance the antitumor effect of TMZ. However, the expression of KAP1 and γH2AX was unaltered by 2-FPA treatment (Supplementary Fig. **6**). We also assessed γH2AX expression after combination treatment involving 2-FPA and TMZ. In A172 and U251 cells, the expression of γH2AX appeared to be higher in the TMZ alone and 2-FPA combined with TMZ groups than in the DMSO and 2-FPA monotreatment groups. However, between TMZ monotherapy and combination treatment, the expression did not show significant differences. In U87 cells, the combination of 2-FPA and TMZ induced higher γH2AX expression (Supplementary Fig. **6B**), indicating that combination treatment induced more DNA damage than DMSO, 2-FPA monotherapy, or TMZ monotherapy in U87 cells. This suggests that 2-FPA does not induce DNA damage in glioma cell lines, but that combination treatment might induce more DNA double-strand damage.

We also assessed whether the observed decreases in p-ERK and unmethylated *MGMT* promoter are related to ACSLs. On the basis of the studies on ACSLs conducted to date, the evidence is scarce to support the contention that either ERK phosphorylation or *MGMT* promoter methylation is downstream of ACSL inhibition. Thus, the inhibition of p-ERK and methylation of the unmethylated *MGMT* promoter appear to be independent events unrelated to ACSLs.

2-FPA was selected from HTS of 1301 compounds and drugs. Although it has been reported to be a fatty acid inhibitor, the safety of 2-FPA for normal brain cells has not yet been revealed. To our knowledge, there are no other reports mentioning the effect of 2-FPA on other types of cancers. According to a previous study, even 0.28 mM of D, L-alpha-FPA showed minor suppressive effects on the cell growth of fibroblasts but no changes in cell morphology [12]. In our study, the highest concentration used was 20 μM. This suggests that the concentration of 2-FPA used in this study would not have deleterious effects on normal cells. Our findings suggest that 2-FPA could be a new potential candidate for clinical applications against glioma, in addition to enhancing the efficacy of TMZ in cases of resistance. Future studies examining the effects of 2-FPA with TMZ *in vivo* are warranted to confirm these preliminary findings.

CONCLUSION

In summary, we identified 2-FPA as a novel potential therapeutic agent against GSCs and glioma cells which functions through the inactivation of ERK and MMP-2, as well as the suppression of the stem cell phenotype. 2-FPA can also enhance TMZ efficacy against GBM by enhancing *MGMT* promoter methylation status, suggesting that combination therapy might be one strategy to improve TMZ efficacy and overcome resistance.

AUTHORS' CONTRIBUTIONS

The authors contributed to the study and manuscript preparation as follows. Conception and design: MN. Acquisition of data: SJ. Analysis and interpretation of data: MN, SJ. Drafting article: SJ, TF. Critically revising the article: all authors. Reviewed the final version of the manuscript and approved it for submission: all authors. Study supervision: MN.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

All materials and protocols were approved by human genome/genetic analysis research ethics committee (Approval Number: 209), and medical ethics committee (Approval Number: 2080, 2188) of Kanazawa University, Kanazawa, Japan.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

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CONFLICT OF INTEREST

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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