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Methionine restriction of glioma does not induce MGMT and greatly improves temozolomide efficacy in an orthotopic nude-mouse model: A potential curable approach to a clinically-incurable disease

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ABSTRACT

Glioma is a highly recalcitrant disease with a 5-year survival of 6.8 %. Temozolomide (TMZ), first-line therapy for glioma, is more effective in O^6-methylguanine-DNA methyltransferase (MGMT)-negative gliomas than in MGMT-positive gliomas as MGMT confers resistance to TMZ. Methionine restriction is effective for many cancers in mouse models including glioma. The concern is that methionine restriction could induce MGMT by decreasing DNA methylation and confer resistance to TMZ. In the present study, we investigated the efficacy of combining methionine restriction with TMZ for the treatment of MGMT-negative glioma, and whether methionine restriction induced MGMT. Human MGMT-negative U87 glioma cells were used to determine the efficacy of TMZ combined with methionine restriction. Recombinant methioninase (rMETase) inhibited U87 glioma growth without induction of MGMT in vitro. The combination of rMETase and TMZ inhibited U87 cell proliferation more than either agent alone in vitro. In the orthotopic nude-mouse model, the combination of TMZ and a methionine-deficient diet was much more effective than TMZ alone: two mice out of five were cured of glioma by the combination. No mice died during the treatment period. Methionine restriction enhanced the efficacy of TMZ in MGMT-negative glioma without inducing MGMT, demonstrating potential clinical promise for improved outcome of a currently incurable disease.

1. Introduction

Glioma is the most prevalent and recalcitrant malignant brain and central-nervous tumor, accounting for half of all malignant brain tumors in the United States [1]. The incidence of glioma is low, at 3.22 per 100,000 people, but mortality is high; the 5-year survival rate is only 6.8 % [2].

Temozolomide (TMZ), an alkylating agent, is first-line chemotherapy for glioma. TMZ is more effective in patients whose tumors do not express the DNA-repair protein O^6-methylguanine-DNA methyltransferase (MGMT) than in those whose tumors do express it [3]. MGMT expression is thought to be suppressed by promoter methylation of the *MGMT* gene, since approximately 45 % of glioma patients have promoter methylation in their tumors [3].

All cancer types are addicted to methionine and is termed the Hoffman effect [4–9]. A number of studies [10–16] have shown that glioma cells are methionine addicted which was first discovered 50 years ago by us [4]. Although methionine addiction is an attractive target to treat glioma and other cancers in combination with chemotherapy [16,17], it was thought that methionine restriction would induce MGMT in cells [18]. Methionine restriction, including with recombinant methioninase, is synergistic with many cancer-chemotherapy drugs. Since methionine restriction selectively arrests cancer cells in the S/G2-phase of the cell cycle [19–22,35,36], alkylating agents similar to TMZ showed synergistic efficacy when used in combination with methionine restriction on mouse models of brain cancer [16,23].

In the present study, we determined the efficacy of methionine restriction in combination with TMZ on a MGMT-negative glioma in vitro and vivo, and found that methionine restriction greatly improved TMZ

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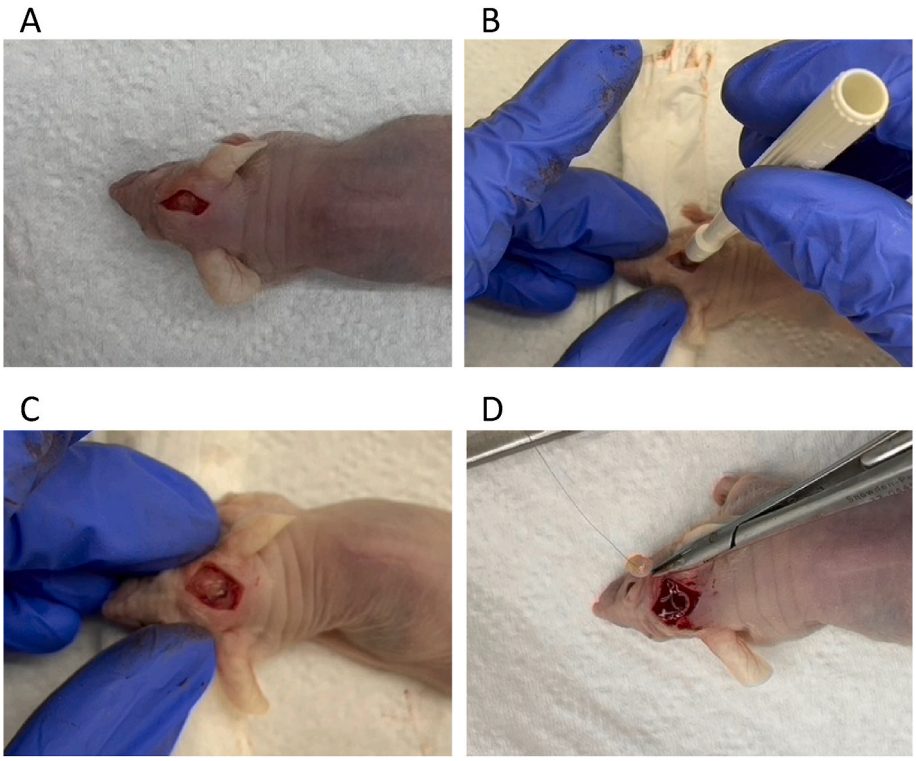

Fig. 1. Tumor transplantation procedure into the brain. A. Opening a skin flap over the brain. B. Making a hole in the cranial bone. C. Removal of the cranial bone. D. Suturing a U87-GFP tumor fragment to the brain.

efficacy on an MGMT-negative glioma, without induction of MGMT.

2. Material and methods

2.1. Cell culture

Human glioma cell lines U87 and LN18 were obtained from the American Type Culture Collection (Manassas, VA, USA). A green fluorescent protein (GFP)-containing lentivirus was transfected into U87 cells as previously described [24]. Dulbecco's modified Eagle's medium (DMEM), with 10 % fetal bovine serum (FBS) and 100 IU/ml of penicillin/streptomycin, was used to cultivate the cells.

2.2. Recombinant methioninase (rMETase) production and formulation

Recombinant methioninase (rMETase) was produced by fermenting recombinant *Escherichia coli* transformed with the methioninase gene from *Pseudomonas putida*. rMETase was purified using a 60-degree thermal step, polyethylene glycol precipitation, and diethylaminoethyl (DEAE)-Sepharose FF column chromatography [25,26].

2.3. Effect of rMETase and temozolomide on the viability of U87 cells

The Cell Counting Kit-8 containing the WST-8 reagent (Dojindo Laboratory, Kumamoto, Japan) was used to count viable cells in culture. Cells were grown in 96-well plates by seeding 5.5×10^3 U87 cells per well in the medium described above. The next day, cells were treated with rMETase concentrations ranging from 0.01 U/ml to 8 U/ml and/or TMZ concentrations ranging from 12.5 μM to 800 μM. TMZ was obtained from MedChemExpress (Monmouth Junction, NJ, USA). Cell viability was measured after 72 h of treatment. IC_{50} values and sensitivity curves were calculated using ImageJ version 1.53 (National Institutes of Health, Bethesda, Maryland, USA). After calculating the IC_{50} of rMETase and TMZ, the synergistic efficacy of the combination of the drugs was examined using the IC_{50} concentration of each drug. Each experiment was conducted three times, in triplicate.

2.4. Immunoblotting for determination of MGMT expression

U87 cells (1.5×10^6) were cultured in 100 mm dishes in DMEM overnight. The following day, the cells were rinsed in PBS, and medium, with or without rMETase at its IC_{50} value, was added. The U87 cells were incubated with the rMETase-containing medium for 3 days (72 h) or 9 days (216 h). At those time points the cells were lysed, and protein was extracted, using the RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Waltham, MA, USA) with the 1 % Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific).

LN18 cells were used as an MGMT-positive control. LN18 cells (3.0×10^6) were cultured in 100 mm dishes in DMEM and treated with rMETase similar to the U87 cells as described above. Protein extraction was performed 3 days (72 h) or 9 days (216 h) after the start of rMETase treatment.

Protein-extract samples were placed on 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gels for electrophoresis and transferred to 0.45 μm polyvinylidene difluoride membranes (GE10600023; GE Healthcare, Chicago, Illinois, USA) after electrophoresis. The membranes were blocked with Bullet Blocking One for Western Blotting (Nakalai Tesque, Kyoto, Japan). Antibodies specific for MGMT (ab126770, 1:10,000; Abcam, Cambridge, United Kingdom) and for β-actin (20536-1-AP, 1:1500; Proteintech, Rosemont, Illinois, United States) were used. β-actin was used as a loading control. Horseradish peroxidase-conjugated anti-rabbit IgG (SA00001-2, 1:20,000; Proteintech, Rosemont, IL, USA) was used as a secondary antibody. A UVP ChemStudio instrument (Analytik Jena, Upland, CA, USA) was used to visualize immunoreactivity with the Clarity Western ECL Substrate (Bio-Rad laboratories, Hercules, CA, USA).

2.5. Mice

Female athymic (nu/nu) mice, aged 4–6 weeks (AntiCancer Inc., San

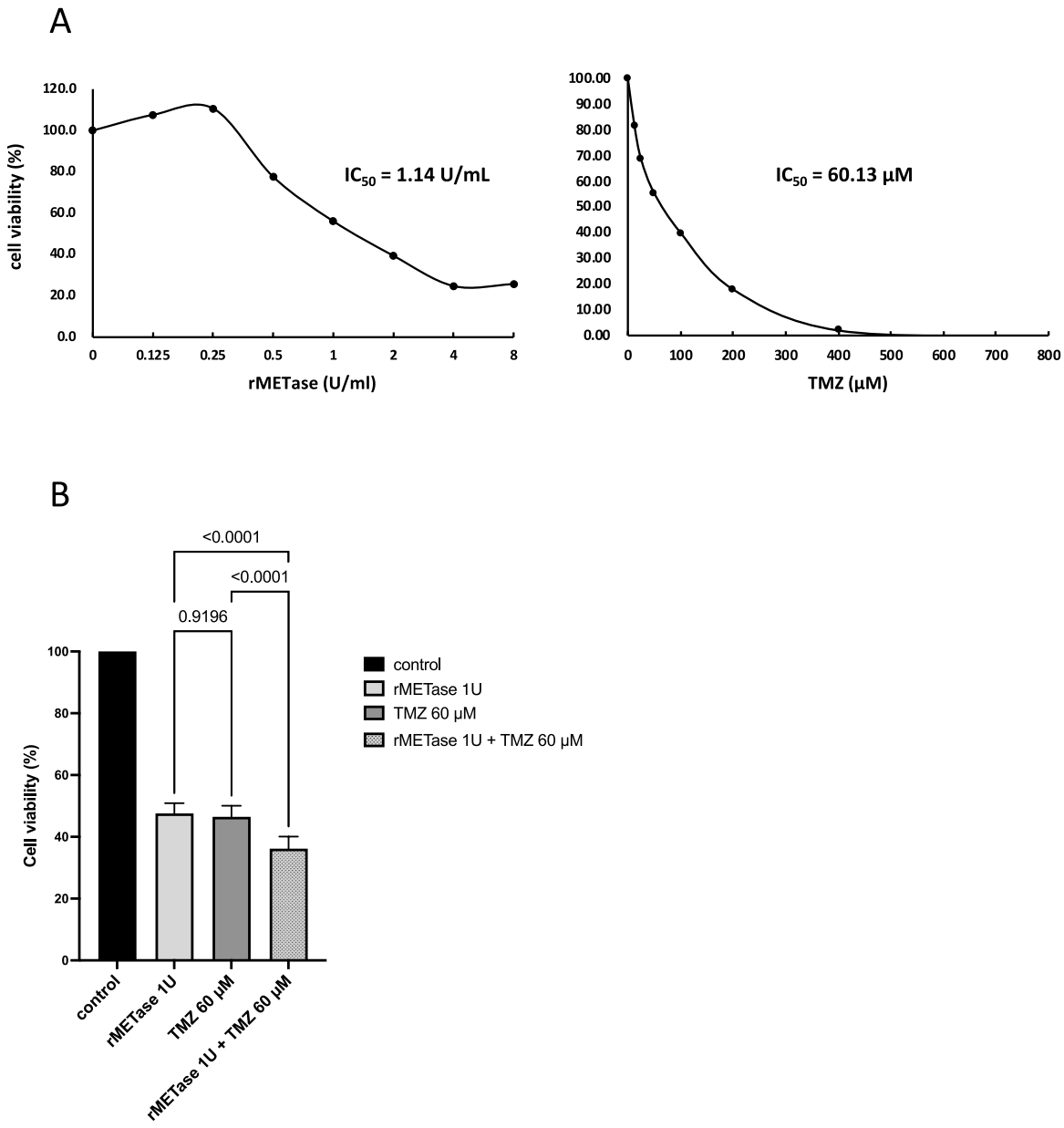

Fig. 2. A. IC_{50} of TMZ and rMETase on U87 cells. B. The combination of rMETase and temozolomide (TMZ) is more effective on U87 cells in vitro than either agent alone.

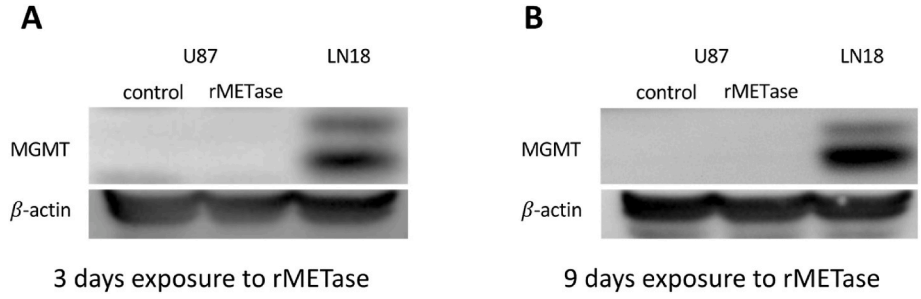

Fig. 3. Immune blotting of MGMT. A. U87 and LN18 cells were treated with rMETase for 3 days. The control lane was derived from untreated U87 cells. B. U87 cells and LN18 cells were treated with rMETase for 9 days. LN18 cells were used for positive control; β-actin was used as a loading control.

Diego, CA, USA), were used in the present study. Mice were confined in a barrier facility equipped with a HEPA-filtered rack and 12 h light/dark cycles. During this study, mice were fed an autoclaved laboratory rodent diet. The AntiCancer Institutional Animal Care and Use Committee's ethical committee approved the present mouse studies. All experiments were conducted according to Animal Research: Reporting of In Vivo

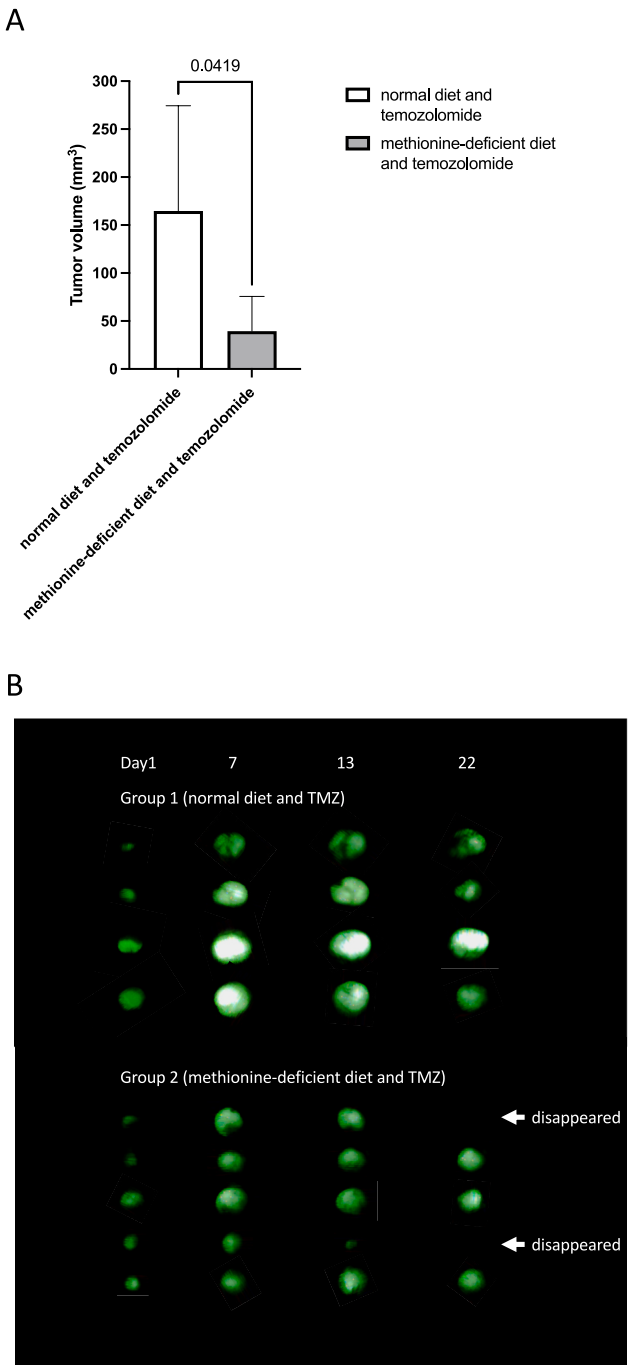

Fig. 4. Efficacy of TMZ and a methionine-restricted diet on an orthotopic mouse model of U87-GFP glioma. A. Tumor volume at day 22. B. Tumor size change during the treatment periods. GFP fluorescence was visualized with the FluorVivo version 2.0.

Experiments (ARRIVE) 2.0 criteria [27].

2.6. Orthotopic glioma nude-mouse model

An orthotopic mouse model of human glioma was established using surgical orthotopic implantation (SOI) as described previously [28]. U87-GFP cells (1×10^6) were first subcutaneously injected in the flank of nude mice. The tumors were harvested 2 weeks after injection, when the tumor grew to 200–300 mm^3. After removing necrotic tissue, viable tissue was cut with a scissors and minced into 4 mm^3 fragments. Mice were anesthetized by injection of 0.02 ml 50 % ketamine, 38 % xylazine, and 12 % acepromazine maleate. A 1 cm long incision along the midline of the nude-mouse scalp (Fig. 1A) was made using a skin biopsy punch (Acuderm Inc.), and then a 4 mm diameter craniotomy was made to expose the parietal bone (Fig. 1B). The bone fragment was removed carefully in order not to injure the meninges and brain tissue (Fig. 1C). A skin flap in the scalp was made in order to image the U87-GFP tumor growing in the brain (Fig. 1D) as previously described [29].

2.7. Temozolomide treatment and methionine restriction in vivo

Ten of 20 mice transplanted with U87 into the brain were chosen and divided into two groups of five mice. Group 1 mice were fed a normal diet and gavaged with TMZ (25 mg/kg, daily) for 5 days followed by 16 days of rest. Group 2 mice were fed a methionine- and choline-deficient diet and gavaged with TMZ as in Group 1. Tumor size was determined using GFP fluorescence imaging, which was performed every 3 days until day 22. Fluorescent tumors were imaged with the FluorVivo version 2.0 (INDEC BioSystems, Santa Clara, CA, USA) Tumor size (mm^3) was calculated with the following formula: length (mm) × width (mm) × width (mm) × 1/2.

2.8. Statistics

All statistical analyses were performed using GraphPad Prism 9.4.0 (GraphPad Software, Inc., San Diego). The unpaired *t*-test was used for the parametric group comparison test. Mean and standard deviation are used to represent all data. A p-value $\leqq 0.05$ is considered significant.

3. Results

3.1. Methionine restriction using rMETase and TMZ inhibited MGMT-negative U87 cell growth in vitro and their combination was significantly more effective than either agent alone

We first evaluated the sensitivity to rMETase and TMZ of U87 cells in vitro, and IC_{50} values were calculated. The IC_{50} doses of rMETase and TMZ were 1.14 U/ml and 60.13 μM, respectively (Fig. 2A). We then evaluated the combination efficacy of rMETase and TMZ. The concentrations used were 1 U/mL for rMETase and 60 μM for TMZ, determined from their respective IC_{50} values. The combination of rMETase and TMZ inhibited U87 cell growth significantly more compared to rMETase or TMZ alone ($p < 0.0001$) (Fig. 2B).

3.2. MGMT expression was not induced by methionine restriction using rMETase

MGMT expression of U87 and LN18 cells was determined after treatment with rMETase for 3 days or 9 days in vitro. rMETase concentrations were chosen by their IC_{50} concentrations, which are 1.1 U/ml for U87 and 1.5 U/ml for LN18. MGMT expression was not detectable at either 3 days or 9 days after rMETase treatment of U87 cells. MGMT-expressing LN18 cells were used as a positive control (Fig. 3).

3.3. Methionine restriction greatly increased TMZ efficacy on the orthotopic mouse model of U87 glioma

In the orthotopic model of U87 glioma, the combination of TMZ and the methionine- and a choline-deficient diet (Group 2) significantly increased the inhibition of tumor growth compared to TMZ alone (Group 1). On day 22, the tumor size of the mice treated with TMZ and the methionine-deficient diet was significantly smaller than the mice treated only with TMZ ($p = 0.0419$) (Fig. 4). In the mice treated with the combination of TMZ and methionine-choline-deficient diet, 2 of 5 mice were cured.

3.4. No mice died in the TMZ and TMZ + methionine-deficient diet group

None of the 5 mice in either the TMZ or TMZ + methionine-deficient diet group died during the treatment period.

4. Discussion

Glioma is perhaps the most recalcitrant of all cancers, and is incurable with a 5-year survival of only 6.8 %. TMZ is first-line therapy for glioma but is not sufficiently effective. The present report demonstrates that methionine restriction greatly improves TMZ efficacy, resulting in a cure rate of 40 %.

MGMT expression is thought to be regulated by the methylation of the CpG island in the promoter region of MGMT [30,31]. For example, methionine restriction has been shown to alter DNA methylation status, including global DNA methylation [32], as well as gene-specific methylation, including LINE-1 methylation [33,34].

Although older and as well as recent studies have shown that gliomas are methionine addicted [10–16], it was thought that methionine-restriction targeting of glioma may therefore induce MGMT activity by reducing its gene-promotor methylation and reduce the efficacy of TMZ. The present study, however, showed that methionine restriction did not induce MGMT expression in MGMT-negative U87 glioma. Therefore, methionine restriction greatly improved the efficacy of TMZ. Notably, 2 of 5 mice were cured of glioma due to combining methionine restriction with TMZ. TMZ is effective for MGMT-negative glioma, but it is almost impossible to achieve a cure in the clinic. Our results are promising for increasing the efficacy of TMZ for MGMT-negative glioma by combining methionine restriction with TMZ, with potential for cure in the clinic.

Data availability

Original data are available from the authors upon request.

CRediT authorship contribution statement

Yutaro Kubota: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Writing – original draft. **Yusuke Aoki:** Investigation. **Noriyuki Masaki:** Investigation. **Koya Obara:** Investigation. **Kazuyuki Hamada:** Methodology, Supervision. **Qinghong Han:** Methodology, Supervision. **Michael Bouvet:** Methodology, Supervision. **Takuya Tsunoda:** Supervision. **Robert M. Hoffman:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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