CHAPTER 4

Hydroxyurea sensitizes glioblastoma to temozolomide

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ABSTRACT

Glioblastoma (GBM) is the most common and most aggressive primary malignant brain tumor. Standard-of-care treatment involves radiation, chemotherapy (temozolomide; TMZ), and maximal surgical resection of the tumor. The 5-year survival rate of patients with GBM is <10%, a colossal failure that has been partially attributed to intrinsic and/or acquired resistance to TMZ. Here we sought to identify therapeutic agents that could enhance the TMZ effect in GBM cells. Through drug screening, we identified hydroxyurea (HU), an FDA-approved drug, to sensitize GBM cells to TMZ. HU was evaluated as TMZ sensitizer in both newly diagnosed GBM as well as recurrent, TMZ-resistant, tumors. We employed cells obtained from patient tumor tissues with different O6-methylguanine methyl transferase (MGMT) promoter methylation status, which after intracranial injection infiltrate the brain of mice similar to GBM in patients. HU synergized with TMZ in vitro and in vivo, to inhibit tumor growth of both newly diagnosed and recurrent tumors, irrespective of the MGMT promoter methylation status. HU might act specifically on the S-phase of the cell cycle by inhibiting the enzyme ribonucleotide reductase, since knockdown of RRM2 (ribonucleotide reductase M2) exerted a similar effect as HU in combination with TMZ on tumor growth both in vitro and in vivo. Although HU has been previously evaluated in malignant glioma patients in combination with radiation or cytotoxic chemotherapy, and has shown limited efficacy, it was never evaluated in combination with TMZ. Altogether, we demonstrate preclinical efficacy and safety of HU in combination with TMZ for treatment of glioblastoma. These results warrant further evaluation of the combination of HU and TMZ in a clinical setting.
INTRODUCTION

Gliomas account for about 60% of all primary central nervous system tumors. Glioblastoma (GBM or grade IV glioma), which comprises 51.2% of all gliomas, is the most malignant form. Over the last two decades, the major breakthrough in the treatment for GBM has been the addition of the DNA alkylating agent temozolomide (TMZ) to the standard of care including surgery and radiation, yielding a modest increase in the median survival from 12.1 months to 14.6 months. An updated survival data analysis from a randomized phase III study revealed an increase in the overall 2-year survival from 10.9% to 27.2% in patients receiving the combined therapy. Despite this success, 90% of patients receiving both TMZ and radiation die within 5 years, a colossal failure that has been partially attributed to drug resistance.

Drug resistance can generally be categorized as either acquired or intrinsic which, on a molecular level, share several common foundations. One of the major predictors of the response of GBM to TMZ is the intrinsic MGMT (O6-methylguanine methyltransferase) promoter methylation status. TMZ induces methylation of guanine at O6 position, a change that causes a futile cycle of attempted DNA repair, and results in cell apoptosis. MGMT removes the DNA adduct caused by the alkylating agent, resulting in resistance to TMZ therapy. Thus, patients whose tumors have transcriptional silencing of the MGMT gene, mediated by promoter methylation (which occurs in approximately half of GBM tumors), are more likely to benefit from the addition of TMZ. Given that all GBMs recur to a tumor lesion with acquired resistance to TMZ, ultimately leading to patient death, studies are underway to unravel the molecular changes that occur during treatment and that characterize the therapy-resistant recurrences. A recent phase II clinical trial on recurrent GBM with dose-intense TMZ showed marginal tumor response and progression-free survival, independent of the MGMT promoter methylation status. Thus, other mechanisms of acquired resistance are likely to be involved. Recently, large-scale cancer genome sequencing analysis of malignant gliomas identified a hypermutation phenotype and somatic truncating/inactivating mutations in the MSH6 mismatch repair gene in recurrent (post-TMZ) GBM, particularly those growing more rapidly during TMZ therapy. Loss of MSH6 was found to occur in a subset of recurred GBM and was associated with tumor progression, mediating TMZ resistance. These studies lead us to believe that novel therapies that could overcome both intrinsic and acquired TMZ resistance in GBM could potentially lead to a therapeutic benefit and an increase in GBM patient survival.

Here, we sought to identify therapeutic agents that could enhance the TMZ response in GBM. Through drug screening of known anti-cancer agents on TMZ-resistant GBM cells, we identified hydroxyurea (HU), an FDA-approved drug, to sensitize TMZ-resistant GBM cells to TMZ, both in culture and in primary GBM in vivo intracranial models irrespective of their MGMT promoter methylation status.
MATERIAL AND METHODS

Cells
Human glioblastoma cell lines Hs683, U87, and LNZ308 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), and antibiotics (100 U of penicillin, 0.1 mg/mL streptomycin) at 37°C and 5% CO2 in a humidified incubator. The generation of TMZ-resistant subclones was previously described10. Briefly, parental cells (Hs683, U87, and LNZ308) were treated twice a week in duplicate with a clinically relevant concentration of TMZ (33 μM). Exposure to TMZ was continued for multiple weeks (10-18 weeks), until two individual resistant subclones were generated of each parental glioblastoma cell line.

Drugs
TMZ and HU (both Sigma) were dissolved in dimethyl sulfoxide (DMSO) to prepare a stock concentration of 100 mM, which was further diluted in 5% dextrose (pH=4) for in vivo experiments. The RNR inhibitors fludarabine or gemcitabine were prepared similar as HU and TMZ.

GBM stem-like cells
Primary GBM cells with different MGMT promoter methylation status were derived from surgical specimens obtained from both newly diagnosed GBMs and recurrent tumors under an approved IRB from the Massachusetts General hospital. The cells were dissociated and grown as neurospheres in serum-free Neurobasal medium supplemented with 3 mM L-glutamine, N2 (1:100), B27 (1:50), 50 μg/mL primocin, 2 μg/mL heparin (all from Life Technologies, Carlsbad, CA), 20 ng/mL human recombinant EGF (R&D Systems, Minneapolis, MN), and 20 ng/mL human recombinant bFGF-2 (Peprotech, Rocky Hill, NJ) (NBM E/F20)16,18. Sphere-like structures usually started to form after one or two days. For passaging, spheres were dissociated using NeuroCult® chemical dissociation kit (Stemcell Technologies, Vancouver, Canada).

Lentivirus vectors
The Gluc cDNA14 and the GFP expression cassette separated by an internal ribosomal entry site (IRES) element was cloned into a lentivirus vector under the control of the strong constitutive cytomegalovirus (CMV) promoter. A similar vector was generated to express Fluc and mCherry14. Lentivirus vector stocks were produced as previously described14. Vectors were titered based on fluorescent protein expression as transducing units (tu) with titers usually around 10⁸ tu/ml. Different GBM cell types were infected with these lentivirus vectors at an MOI of 10 by adding the vector directly to the cells, which resulted in >90% transduction efficiency in GBM cells and spheres13,14.
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Gluc assay
Cells were plated in clear-bottom, black 96-well plates at a density of 3000-5000 cells/well. To monitor cell growth, aliquots of cell-free conditioned medium (10µL) were transferred to a white 96-well plate. Gluc activity was determined using FlexStation3 microplate reader (Molecular Device, Sunnyvale, CA) under “Flex” mode after adding 100 µL of 40 µM coelenterazine (Nanolight, Pinetop, AZ), which is dissolved in acidified methanol and further diluted in phosphate buffered saline (PBS). To monitor tumor growth in vivo, 5 µL of blood was withdrawn from the tail vein and immediately mixed with 1 µl of 20 mM EDTA. Gluc activity in blood was measured after addition of 100 µL of 100 µM coelenterazine using a plate luminometer (Dynex, Richfield, MN).

Secondary sphere formation assay
To investigate the capacity of single cells to generate new neurospheres, GBM cells were plated at a concentration of 1000 cells/well in 500 µl NBM E/F20 in a 48-well plate and treated for five consecutive days with HU and/or TMZ. Recovery of the neurospheres was determined 9 days after start of treatment by counting the number of neurospheres using phase-contrast images of the spheres (40x magnification). Subsequently, neurospheres were dissociated and secondary sphere formation was determined 14 days after start of treatment by counting the number of spheres.

RNAi (knockdown RRM2)
An shRNA plasmid against human RRM2 gene and a scrambled control (both Sigma) were packaged into a lentivirus envelope. U87P, U87R1, MGG6, and MGG23 cells were transduced by adding the lentiviral particles to the cells at an MOI of 10. Subsequently, stably transduced cells were selected for ten days using puromycin. The knockdown of RRM2 was evaluated by real-time quantitative PCR and Western blot analysis.

Apoptosis assay (Annexin V/PI staining)
After treatment (24 hrs), cells were harvested, washed in cold PBS, and resuspended in 1x binding buffer. Subsequently, cells were stained with 1 µl Annexin V-PE-Cy5 and 1 µl propidium iodide (PI), gently vortexed, and incubated for 15 minutes at room temperature in the dark. After incubation, 400 µl 1x binding buffer was added to the cells and the samples were analysed using flow cytometry (Becton Dickinson). Twenty thousand events were measured and results were expressed as the percentage (Annexin V + with PI +/−) of apoptotic cells.

In vivo models
All animal studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care following guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. GBM cells were first
transduced with a lentivirus vector to stably express Fluc. Nude female mice were anesthetized (100 mg/kg ketamine and 5 mg/kg xylazine) and fifty thousands GBM cells were stereotactically injected into the brain using the following coordinates from the bregma in mm: anterior-posterior +0.5 mm, medio-lateral +2.0 mm, dorso-ventral −2.5 mm, as we previously described\textsuperscript{14}. One week after implantation of 50,000 (low amount) or 200,000 (high amount) GBM cells or 50,000 primary GBM cells (dissociated from spheres), mice were randomized into different treatment groups. Tumor growth was monitored over time by \textit{in vivo} Fluc bioluminescence imaging after i.p. injection of 200 mg/kg D-luciferin substrate and acquiring signal using the Xenogen IVIS 200 Imaging System (Caliper Life, Hopkinton, MA) as we previously described\textsuperscript{14}.

**Statistical analysis**

Differences between treatment groups were determined using a two-tailed Student’s \textit{t} test (unpaired), ANOVA and Tukey’s post-hoc test. Survival analysis was conducted by Kaplan-Meier curves, and their comparison was determined by log rank test. \textit{p} values <0.05 were considered significant.

**RESULTS**

**HU sensitizes resistant glioblastoma cells to TMZ \textit{in vitro}**

We previously generated two independent TMZ-resistant subclones from three different glioblastoma cell lines (U87, LNZ308 and Hs683) by long-term exposure (two times per week over 10-18 weeks) to a clinically relevant concentration of TMZ (33 μM), generating U87R1, U87R2, LNZ308R1, LNZ308R2, Hs683R1 and Hs683R2\textsuperscript{10,11}. MGMT methylation and MMR status of the resistant cells was previously assessed and these mechanisms did not seem to play a role in the acquired TMZ resistance obtained in these cells\textsuperscript{10}. These cells together with their parental counterparts were first engineered by a lentivirus vector to stably express the naturally secreted Gaussia luciferase (Gluc) as a reporter for cell viability. The level of Gluc secretion to the conditioned medium is linearly related with respect to cell number and proliferation\textsuperscript{12–14}. In this case, cell viability can be monitored over time by assaying aliquots of conditioned medium from these cells for Gluc activity. Exposure of these cells to TMZ revealed that the three parental glioblastoma lines were sensitive to TMZ in a dose-dependent fashion, while no significant effect was observed on any of the resistant cell lines up to 100 μM (Fig. 1A). Using the U87R1 and U87R2 TMZ-resistant cells and the Gluc high-throughput screening assay which we have recently described\textsuperscript{15}, we screened a small library of known anti-cancer agents in the presence and absence of 100 μM TMZ. After screening and validation, we found the compound HU to sensitize both U87 resistant cell lines to TMZ (Fig. 1B). The same results were obtained when HU was used in combination with TMZ on LNZ308 and Hs683 resistant lines (Fig. 1C and D). FACS analysis using AnnexinV/PI staining confirmed an increase in cell death upon combined therapy in U87 cells (Fig. 1E).
Figure 1. HU sensitizes glioblastoma cells to TMZ in vitro. (A) U87, LNZ308, Hs683 parental cells (P) and resistant clones (R1/R2) were treated with different amounts of TMZ. After four days aliquots of conditioned medium were assayed for Gluc activity using 50 ml of 2mg/ml coelenterazine. (B) U87 and resistant clones R1/R2, (C) LNZ308 and resistant clones R1/R2, and (D) Hs683 and resistant clones R1/R2 were treated with TMZ and/or 100 μM HU. Cell viability was assessed by the Gluc assay. (E) Cell death after treatment (control, TMZ, HU, HU+TMZ) determined by FACS analysis using AnnexinV/PI staining of U87 cells.
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HU sensitizes resistant glioblastoma cells to TMZ in vivo

We then validated these data in an in vivo intracranial model using U87 and U87R1 cells. These cells were first transduced with a lentivirus vector to stably express firefly luciferase (Fluc), a reporter which can be used to track tumor growth in vivo. Fifty thousands of these cells were intracranially injected in the brain of nude mice as previously described. One week post-tumor implantation, mice were randomized into four different groups receiving: (1) DMSO vehicle control; (2) intraperitoneal (i.p.) injection of 5 mg/kg TMZ; (3) 50 mg/kg HU i.p.; (4) combination of TMZ+HU. Tumor growth was monitored over time by in vivo Fluc bioluminescence imaging using a cooled CCD camera. HU alone had no significant effect on U87 or U87R1 tumor growth and mice survival. TMZ alone had a moderate effect on parental U87 tumors and survival rate compared to vehicle-treated group (median survival = 24.5 days vs. 19 days, respectively, p=0.02), but not on U87R1 tumors (median survival = 11 days for both groups, p=0.36). On the other hand, combination of both HU+TMZ slowed down both U87 and U87R1 tumor growth, leading to a significant increase in mice survival (median survival = 51 days, p=0.02 and 33 days, p=0.0002, respectively; Fig. 2A and B). In another experiment, we challenged this therapeutic strategy by implanting higher number of U87R1 cells (200,000 cells) and waited two weeks before starting the combined therapy to allow the tumor to grow to a large size. Based on Fluc imaging, tumors had reached saturation signal and mice were expected to die within a few days. As expected, all control mice died within 2-4 days while the HU+TMZ-treated mice survived for another 10 days showing the efficiency of this combined therapy (median survival = 16 and 22 days for TMZ and HU+TMZ group, respectively, p=0.003; Fig. 2C). We then tested the effect of HU on U87R1 cells in combination with standard of care (radiation and TMZ) using the same intracranial model (50,000 U87R1 cells implanted and therapy initiated one week later). The triple therapy yielded an enhanced therapeutic effect on U87R1 tumors and increased survival rate, with 50% of mice remaining alive 48 days after implantation of the GBM cells (median survival for control, 23 days; radiation only, 34 days; HU only, 27.5 days; TMZ only, 23.5 days; radiation+TMZ, 29.5 days; radiation+HU+TMZ, 48 days, p=0.0004; Fig. 2D).

HU sensitizes primary GBM cells to TMZ irrespective of the MGMT promoter methylation status

Next, we determined the effect of HU on TMZ response in primary GBM cells. We obtained GBM cells with different MGMT promoter methylation status from newly diagnosed and recurrent patient tumor sections and grew them as neural spheres in stem cell medium. Cells cultured this way retain the phenotype and genotype of primary tumors including MGMT promoter methylation status and only cells with stem-like properties (e.g. Nestin/Sox2 expression) will form spheres and grow under these conditions. GBM stem cells have been proposed to be the source of
Figure 2. HU sensitizes glioblastoma cells to TMZ in vivo. (A-B) U87 or U87R1 cells expressing Fluc were implanted in the brain of nude mice. One week later, mice were divided in four groups (n=10-20/group) and treated with either DMSO vehicle, HU, TMZ or TMZ+HU. Tumor growth was monitored over time by Fluc imaging and survival was recorded generating Kaplan-Meier plots. (C) 200,000 U87R1 cells expressing Fluc were implanted in the brain of nude mice. Two weeks later, mice were divided in two groups (n=10/group) and treated with either TMZ or TMZ+HU. Survival was recorded. (D) U87R1 cells expressing Fluc were implanted in the brain of nude mice. One week later, mice were divided in four groups (n=10-20/group) and treated with either DMSO vehicle, radiation (R; 3 Gy), HU, TMZ, R+TMZ or R+TMZ+HU. Tumor growth was monitored over time by Fluc imaging and survival was recorded generating Kaplan-Meier plots.
tumor recurrence and patient death\textsuperscript{20}, are resistant to conventional therapy\textsuperscript{21}, and can recapitulate a phenocopy of the original tumor upon implantation in nude mice\textsuperscript{22}. We applied the HU+TMZ (30 μM of each drug) combined therapy on GBM neural spheres from newly diagnosed patients with methylated and unmethylated MGMT promoter as well as spheres from recurrent tumors. In culture, the combined HU+TMZ yielded an enhanced therapeutic effect on sphere formation, growth, and recovery, irrespective of their MGMT methylation status of both newly diagnosed and recurrent tumors (secondary sphere formation, TMZ alone vs. HU+TMZ, \( p<0.001 \), respectively; Fig. 3A and B).

\textbf{In vivo effect of HU in combination with TMZ on primary GBM}

Primary GBM8 cells\textsuperscript{18} from newly diagnosed tumor with methylated MGMT\textsuperscript{16} were grown in stem cell medium as neural spheres. These cells were transduced with a lentivirus vector expressing Fluc and mCherry\textsuperscript{14} at a multiplicity of infection (MOI) of 10 yielding >90% infection efficiency. Upon intracranial injection, these cells formed tumors and infiltrated the brain of nude mice similar to human tumors (Fig. 4A-C). We used this model to test the efficacy of HU in combination with TMZ on primary GBM. Four weeks after implantation of 50,000 GBM8 cells dissociated from spheres, when tumors started to rapidly grow (as observed by Fluc imaging), mice were divided into two groups which received DMSO vehicle (\( n=6 \)) or i.p. injection of 30 mg/kg TMZ (\( n=24 \)) four days/week over two weeks. All mice in the DMSO-treated group died by week seven after implantation of the GBM cells, whereas these tumors carrying methylated MGMT promoter responded very well to TMZ as expected (Fig. 4D-F). Mice were then left off TMZ to allow for GBM tumors to recur potentially becoming resistant to TMZ, recapitulating the patient scenario. At this point, the TMZ group was divided into four subgroups (\( n=6/\text{group} \)), which received DMSO, HU, TMZ or HU+TMZ four days/week over three weeks. The second round of TMZ treatment had a moderate effect on tumor volume but did not result in a statistically significant increase in survival (median survival = 12.5, 14, 14 weeks for DMSO, HU, TMZ group after GBM8 implantation, respectively). On the other hand, the combined HU+TMZ therapy had a remarkable and significant effect on both tumor growth and survival rate, with 80% of mice surviving over six weeks (compared to second round of TMZ alone) and remained tumor-free (median survival >22 weeks after GBM8 implantation, \( p<0.0001 \)) (Fig. 4F). We then tested this combined therapy on GBM tumors carrying unmethylated MGMT promoter (MGG23)\textsuperscript{16}, and observed that these tumors also responded very well to the combined HU+TMZ therapy, leading to tumor regression and significant increase in survival rate (median survival = 32.5, 45, 50.5, and 79.5 days for control, TMZ, HU, and TMZ+HU group (\( n=6/\text{group} \)), respectively. \( p<0.0001 \); Fig. 4G-I). Altogether, these data support our hypothesis that HU+TMZ combination could be used to treat newly diagnosed and recurrent GBM tumors irrespective of their MGMT promoter methylation status. Next, we assessed toxicity of this combined therapy by treating mice with either DMSO, TMZ, HU, or HU+TMZ for four consecutive days and
Figure 3. Effect of HU+TMZ on GBM spheres with different MGMT promoter methylation status. (A-B) GBM neural spheres from newly diagnosed GBM tumors with methylated and unmethylated MGMT promoter and spheres from recurrent GBM were plated in a 48-well plate and treated for five days with HU and/or TMZ. Spheres recovery and secondary sphere formation were analyzed for the different treatment strategies.
Figure 4. HU sensitizes primary GBM8 to TMZ in vivo. (A) 5x10^4 GBM8 cells expressing Fluc and mCherry were stereotactically injected into the left midstriatum of nude mice brain as small neurospheres. (B) Tumor growth was monitored by Fluc imaging. (C) At the last imaging point, mice were sacrificed and brains were sectioned and analyzed for mCherry using microscopy. GBM8 infiltrated the brain from the left to the right side via the corpus callosum (cc). White arrow, shows the injection site. Bar, 500 µm. (D) Mice-bearing GBM8-Fluc-mCherry tumors were treated with TMZ or vehicle for two weeks, and then left off TMZ for three weeks. TMZ-treated group was then divided into four subgroups, which received vehicle, HU, TMZ, or HU+TMZ. (E) Tumor growth was
analyzed the blood for white blood cells (WBC), hematocrit (HCT), mean corpuscular volume (MCV), red cell distribution width (RDW), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), red blood cells (RBC), platelets (PLT), and mean platelet volume (MPV). No significant differences were found between treatment groups in comparison to DMSO control (Supplementary Table 1). We also evaluated hematotoxicity on bone marrow by preparing bone marrow smears and counting viable cells from untreated and TMZ+HU-treated naïve mice as above. Under our experimental conditions, no apparent toxicity was observed with this combined therapy (Fig. 4J).

**Mechanism of action of HU**

We sought to understand the mechanism by which HU is synergizing with TMZ. Since HU is known to target ribonucleotide reductase subunit M2 (RRM2), an enzyme involved in nucleotide metabolism, a knockdown experiment was first performed in different cell lines using shRNA against RRM2 (shRRM2) or scrambled control (ShScram) and different concentrations of TMZ. As expected, RRM2 knockdown sensitized all GBM cells including parental U87 (U87P), U87R1, MGG6 (methylated MGMT promoter), and MGG23 (unmethylated MGMT promoter) to TMZ irrespective of MGMT promoter methylation status (Fig. 5A and B). Further, the same sensitizing effect was observed on U87 cells implanted intracranially and expressing shRRM2 (Fig. 5C and D). Other ribonucleotide reductase (RNR) inhibitors were also evaluated to determine if a similar effect as HU is obtained on GBM. Primary GBM cells from recurrent tumors (pGBMrA) expressing Gluc were treated in a 96-well plate with either fludarabine or gemcitabine – classical RNR inhibitors - and cell viability was assessed using the Gluc assay. As expected, TMZ alone had a moderate-to-no effect on these cells. Both RNR inhibitors sensitized the GBM cells to TMZ suggesting that ribonucleotide reductase is a key player in the synergistic effect (Fig. 5E).

**DISCUSSION**

Novel treatment strategies for GBM are urgently needed since outcome for GBM patients remains extremely poor. GBM is the most common and most aggressive primary malignant brain tumor in adults with a 5-year survival rate of <10%2. Standard-of-care treatment involves radiation, chemotherapy (temozolomide; TMZ), and maximal surgical resection of the tumor. Therapy resistance is a significant obstacle in the treatment of this devastating disease and both intrinsic and acquired resistance to TMZ monitored by Fluc imaging once/week. (F) Survival was recorded generating Kaplan-Meier plots. (G) Mice-bearing MGG23-Fluc tumors with unmethylated MGMT promoter were treated with either vehicle (control), TMZ, HU or HU+TMZ. (H) Tumor growth was monitored by Fluc imaging once/week. (I) Survival was recorded generating Kaplan-Meier plots. (J) Mice were treated with DMSO or HU+TMZ for four consecutive days and bone marrow smears were analyzed by microscopy.
Figure 5. Mechanism of HU+TMZ synergism. (A) U87 and resistant clones R1 cells or (B) TMZ-sensitive MGG6 and TMZ-resistant MGG23 primary glioma cultures were infected with lentiviral vector expressing scrambled shRNA (shScram) or shRNA against RRM2 (shRRM2) and treated with 0 - 100 µM TMZ. Cell viability was monitored with alamar blue assay. Data presented as the mean Gluc RLU/mL +/- SD (n=8; **p<0.01 vs. shScram vs. shRRM2 expressing cells by two-way ANOVA). (C) The left forebrains of mice were implanted with 2x10^4 U87 cells expressing Fluc and mCherry and infected with shScram or shRRM. Each group of mice was divided into 2 subgroups, which received an i.p.
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contribute to treatment failure. Finding novel drugs that would enhance TMZ effect in newly diagnosed tumors and/or that would re-sensitize the recurrent resistant tumors to TMZ could provide a major benefit to GBM patients, leading to an increase in the overall survival rate. Here, we identified hydroxyurea (HU) as potential adjuvant therapy for glioblastoma since we found this FDA-approved drug to sensitize GBM cells to TMZ.

HU is a simple organic compound that acts specifically on the S-phase of the cell cycle by inhibiting the enzyme ribonucleotide reductase, thereby hindering the reductive conversion of ribonucleotides to deoxyribonucleotides and thus limiting de novo DNA synthesis. In addition, HU can inhibit the repair of DNA damage induced by chemicals or irradiation, which offers potential synergy between HU and irradiation or DNA-damaging agents. These properties make HU an attractive candidate for cancer therapy as we show here in combination with TMZ for the treatment of GBM. HU is FDA-approved and is being used to treat myeloproliferative diseases, sickle cell anaemia as well as some forms of tumors (such as melanoma, ovarian, squamous cell carcinoma, head and neck carcinoma and brain tumors) in combination with other anti-cancer agents. Although HU has been previously evaluated in malignant gliomas in combination with radiation or cytotoxic chemotherapy and has shown limited efficacy, it was never evaluated with an agent with validated efficacy in GBM such as TMZ. Therefore, this is the first demonstration that HU in combination with TMZ has potential as adjuvant therapy for GBM patients.

Major obstacles for treatment of brain tumors, including GBM, are the blood brain barrier (BBB) and the blood tumor barrier (BTB), which hamper delivery of chemotherapeutics to the brain and/or tumor. An advantage for the use of HU for brain tumors is that it has been reported to efficiently pass the blood brain barrier. In addition, a study in rats has shown that HU can increase the blood brain tumor permeability of certain chemotherapeutics by means of nitric oxide production and therefore could enhance penetration of TMZ to GBM, although a pre-clinical analysis showed that HU had no effect on permeability of Imatinib Mesylate (Gleevec) across the blood-brain barrier; a combination of drugs currently being evaluated for the treatment of GBM. The data presented here indicate that HU can penetrate brain tumors since HU+TMZ therapy showed a robust therapeutic effect in different GBM models. However, whether HU increases the penetration of TMZ to the brain and/or to GBM tumors thereby leading to an enhanced therapeutic effect of TMZ, warrants further evaluation.

injection (3 times per week over 3 weeks) of either DMSO or TMZ (30 mg/kg body weight). In vivo Fluc bioluminescence imaging was performed once/week to monitor tumor growth. Representative images at 0, 10, and 17 days post-TMZ treatment are shown. (D) Tumor growth monitored overtime as described in (C). Data presented as the mean of total flux of Fluc (photons/sec) +/- SD (n=6; **p<0.01 vs. shScram + DMSO; ##p<0.01 vs. shScram or DMSO alone by ANOVA and Tukey’s post-hoc test). (E) Primary GBM cells from a recurrent tumor were treated with different doses of two RNR inhibitors and/or TMZ (100 μM). Three days later, cell viability was monitored by the Gluc assay.
One of the major predictors of the response of GBM to TMZ is the intrinsic MGMT (O6-methylguanine methyl transferase) promoter methylation status$^2$. MGMT activity can remove the DNA adducts caused by TMZ thereby conferring TMZ resistance. Thus, patients with a methylated MGMT promoter are more likely to benefit from TMZ treatment. However, a recent phase II clinical trial on recurrent GBM with dose-intense TMZ showed marginal tumor response and progression-free survival, independent of the MGMT promoter methylation status$^6$. Here, we evaluated HU in combination with TMZ for the treatment of both newly diagnosed GBM as well as recurrent, TMZ-resistant, tumors with different MGMT promoter methylation status. We employed cells obtained from patient tumors and show that HU synergizes with TMZ, on both newly diagnosed and recurrent tumors, irrespective of the MGMT promoter methylation status. This indicates that this treatment combination is not limited to GBM patients with a methylated MGMT promoter. GBMs are currently subdivided into four molecular distinct subtypes, classical, neural, proneural, and mesenchymal subtypes$^{36,37}$. It would be of interest to determine if a combination of HU and TMZ would be beneficial for all these subtypes or if patients with a certain subtype would benefit more from this treatment regimen.

In conclusion, this study provides the first demonstration for the use of HU as adjuvant therapy for GBM in combination with TMZ. Since HU is FDA-approved and has been used before in the treatment of cancer including gliomas, it is relatively easily translated to the clinic. A phase I clinical trial of HU and TMZ in patients with recurrent glioblastoma should be initiated to identify a maximum tolerated dose (MTD) for this combination and to explore the quantitative (frequency, duration) and qualitative (organ specific) nature of acceptable and unacceptable toxicities. In addition, preliminary information on the efficacy of this regimen could be obtained. Altogether, the combination of HU and TMZ as adjuvant therapy for GBM patients could provide a major benefit to GBM patients, leading to an increase in the overall survival rate.

REFERENCES
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17. Lee, J. et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 9, 391–403 (2006).


**SUPPLEMENTARY INFORMATION**

**Supplementary Table 1.** Toxicity assessment of the treatment by treating mice with DMSO, TMZ, HU, or HU+TMZ for four consecutive days

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<td>21.4±1.4</td>
<td>23.7±2.9</td>
<td>29.8±1.4</td>
<td>17.4±2.9</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>42.6±0.6</td>
<td>42.4±0.8</td>
<td>43.5±0.7</td>
<td>42.0±0.2</td>
</tr>
<tr>
<td>RDW (fl)</td>
<td>31.4±1.5</td>
<td>31.0±1.6</td>
<td>32.8±1.6</td>
<td>29.3±0.5</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>19.4±1.0</td>
<td>19.4±1.0</td>
<td>19.7±0.7</td>
<td>18.1±0.2</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>8.0±0.5</td>
<td>8.9±0.9</td>
<td>10.8±0.5</td>
<td>6.7±1.1</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>37.6±0.3</td>
<td>37.7±0.9</td>
<td>36.3±0.2</td>
<td>38.9±0.4</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>16.0±0.2</td>
<td>16.0±0.2</td>
<td>15.8±0.3</td>
<td>16.3±0.2</td>
</tr>
<tr>
<td>RBC (10^6/μl)</td>
<td>5.0±0.3</td>
<td>5.6±0.6</td>
<td>6.9±0.2</td>
<td>4.1±0.7</td>
</tr>
<tr>
<td>PLT (10^3/μl)</td>
<td>184.5±38.2</td>
<td>234.7±28.8</td>
<td>122.0±25.7</td>
<td>162.7±21.6</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>6.2±0.2</td>
<td>6.3±0.4</td>
<td>6.5±0.4</td>
<td>6.2±0.2</td>
</tr>
</tbody>
</table>

We analyzed the blood for white blood cells (WBC), hematocrit (HCT), mean corpuscular volume (MCV), red cell distribution width (RDW), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), red blood cells (RBC), platelets (PLT), and mean platelet volume (MPV).