Inhibition of Histone Deacetylation Potentiates the Evolution of Acquired Temozolomide Resistance Linked to MGMT Upregulation in Glioblastoma Xenografts


Abstract

**Purpose:** The therapeutic benefit of temozolomide in glioblastoma multiforme (GBM) is limited by resistance. The goal of this study was to elucidate mechanisms of temozolomide resistance in GBM.

**Experimental Design:** We developed an *in vivo* GBM model of temozolomide resistance and used paired parental and temozolomide-resistant tumors to define the mechanisms underlying the development of resistance and the influence of histone deacetylation (HDAC) inhibition.

**Results:** Analysis of paired parental and resistant lines showed upregulation of O6-methylguanine-DNA methyltransferase (MGMT) expression in 3 of the 5 resistant xenografts. While no significant change was detected in MGMT promoter methylation between parental and derivative-resistant samples, chromatin immunoprecipitation showed an association between MGMT upregulation and elevated acetylation of lysine 9 of histone H3 (H3K9-ac) and decreased dimethylation (H3K9-me2) in GBM12 and GBM14. In contrast, temozolomide resistance development in GBM22 was not linked to MGMT expression, and both parental and resistant lines had low H3K9-ac and high H3K9-me2 within the MGMT promoter. In the GBM12TMZ-resistant line, MGMT reexpression was accompanied by increased recruitment of SP1, C-JUN, NF-κB, and p300 within the MGMT promoter. Interestingly, combined treatment of GBM12 flank xenografts with temozolomide and the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) favored the evolution of temozolomide resistance by MGMT overexpression as compared with treatment with temozolomide alone.

**Conclusion:** This study shows, for the first time, a unique mechanism of temozolomide resistance development driven by chromatin-mediated MGMT upregulation and highlights the potential for epigenetically directed therapies to influence the mechanisms of resistance development in GBM.

Introduction

Temozolomide is an important component of therapy for glioblastoma multiforme (GBM). Unfortunately, tumor progression while receiving temozolomide therapy occurs in more than 40% of patients (1–4), and progression during second-line therapy is essentially universal (3, 5, 6). Thus, understanding mechanisms of primary and acquired temozolomide resistance is critically important. The DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) and the mismatch repair (MMR) pathway are critical mediators of temozolomide sensitivity (7). MGMT removes cytotoxic O6-methylguanine (O6-MG) lesions induced by temozolomide (7–9). Suppression of MGMT expression, often mediated by MGMT promoter hypermethylation, leads to persistent O6-MG lesions that are mispaired with thymidine during replication. This mispairing subsequently is engaged by futile cycles of MMR that lead to collapsed replication forks and death (3, 7). High MGMT expression and disrupted MMR function are mechanistically linked to temozolomide resistance in multiple tumor models (10, 11), and elevated MGMT expression and/or lack of MGMT promoter hypermethylation in patient tumor specimens is associated with a worse outcome in patients with GBM treated with temozolomide (3, 12, 13). Mutations in MMR genes are rare in primary GBM, but acquired MSH6 mutations are associated with development of temozolomide resistance (14, 15). However, this mechanism only accounts for a small proportion of patients, and thus there is significant interest in delineating other mechanisms of temozolomide resistance.
The limited number of clinically relevant tumor models is a major impediment to evaluating mechanisms of chemoresistance in GBM. Traditional glioma cell lines have been cultured for decades and are genetically distinct from primary human tumors, and engineered models do not adequately recapitulate potential epigenetic mechanisms of resistance. While collections of paired patient tumor samples from diagnosis and recurrence following temozolomide therapy have been reported, most of these samples are paraffin-embedded, and small tissue samples limit the molecular assays that can be applied to these scarce samples. To address the limitations, we subjected primary GBM xenograft lines from the Mayo panel to in vivo selection with escalating temozolomide doses. These paired primary and temozolomide-resistant lines provide unlimited amounts of tissues that can be analyzed to define mechanisms of acquired resistance. Herein, we use this model to establish a unique mechanism of acquired temozolomide resistance linked with chromatin-mediated MGMT upregulation. Furthermore, we show that combined therapy with temozolomide and an HDAC inhibitor promotes this epigenetically driven mechanism of resistance.

Materials and Methods

Establishment of primary and temozolomide-resistant GBM xenograft lines
The Mayo GBM xenograft panel has been previously described. Temozolomide resistance models were developed from mice with established flank tumors treated either with 20 mg/kg/d for 3 days and then 66 mg/kg/d for 3 days after initial tumor regrowth or treated with 66 mg/kg/d for 3 days. The resulting temozolomide-resistant lines were completely resistant to a challenge of 120 mg/kg/d for 5 days. The efficacy of temozolomide in resistant models was evaluated using an orthotopic therapy model. All animal studies were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Translational Relevance
The therapeutic benefit of temozolomide, the only drug known to significantly enhance survival in newly diagnosed patients with glioblastoma multiforme (GBM), is limited by rapid evolution of secondary resistance of which the principal mechanisms are poorly known. In this report, the authors have developed an in vivo model of secondary temozolomide resistance and subsequently showed that histone deacetylase (HDAC) inhibition can influence the mechanism of temozolomide resistance emergence, particularly favoring resistance linked with chromatin-mediated O6-methylguanine-DNA methyltransferase (MGMT) upregulation. With an increasing number of epigenetically targeted therapies being tested in oncology, these results highlight the potential for this class of novel therapeutics to influence resistance emergence of a coadministered cytotoxic agent.

Short-term explant cell cultures
Short-term explant cultures grown in serum-containing media were derived from the parental and resistant flank xenografts as described. Serum-free explant cultures were established as described by others; mechanically disaggregated tumors were plated on laminin-coated flasks overnight in neurobasal serum-free media (StemPro NSC SFM; Invitrogen).

In vitro cytotoxicity assays
Explant cultures were plated in triplicate on 96-well plates and treated with graded concentrations of temozolomide ± 10 μmol/L O6-benzylguanine (O6-BG). After 6 days, samples were analyzed using a CyQUANT Assay (Invitrogen) according to manufacturer’s instructions. For a neurosphere assay, explant cultures in neurobasal media were plated in triplicate at 500 cells per well and treated as above. Intact neurospheres were counted after 14 days.

Analysis of MGMT promoter methylation, mRNA, and protein levels
Tumor samples were analyzed for MGMT promoter methylation by methylation-specific PCR (MS-PCR) and expression by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) as described. The same specimens were processed for Western blotting using the antibodies: MGMT (R & D systems), β-actin (Sigma), and horseradish peroxidase–conjugated to secondary antibodies (Pierce; ref. 23). Bisulfite-modified DNA was evaluated by pyrosequencing using a PyroMark MD system (Qiagen).

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was conducted using the EZ-ChIP Kit (Millipore). Tumor samples were minced, and cross-linking was conducted with 1% formaldehyde, quenched with 0.1 mol/L glycine, and then processed according to manufacturer’s instructions. Antibodies used were anti-acetyl-lysine 9 histone H3 (H3K9-ac) and anti-dimethyl-lysine 9 histone H3 (H3K9-me2) from Millipore, and anti-trimethyl-lysine 27 histone H3 (H3K27-me3) from Cell Signaling Technology. The anti-SP1, -C-JUN, -p300/CBP antibodies were from Santa Cruz Biotechnology. The distal promoter region critical for MGMT silencing by hypermethylation was PCR-amplified with human-specific primer sequences: 5′-GCCCGGATATGCTGG-GAC-3′ (forward) and 5′-GGCAACACCTGGAGG-CAC-3′ (reverse). A 35-cycle PCR was conducted using 60°C annealing temperature. NH Image (http://rsweb.nih.gov/ij/) was used to quantitate PCR bands and relative enrichment was a ratio of the immunoprecipitated signal versus input.
Effect of HDAC inhibitor SAHA on the evolution of temozolomide resistance

Mice with GBM12 tumors were treated with placebo or 50 mg/kg/d temozolomide for 5 days every 28 days for 3 cycles. Tumor volume was measured thrice weekly, and mice with tumors exceeding 1,500 mm³ were euthanized. Tumors from each mouse were flash-frozen and cryopreserved. In a second study, GBM12 xenografts were treated with placebo or temozolomide (50 mg/kg for days 1–5) with or without suberoylanilide hydroxamic acid (SAHA; 100 mg/kg on days 1–7 and 15–21 for every 28 days) for 3 cycles.

Statistical analysis

The log-rank test was used to compare animal survival between placebo- and drug-treated mice. Relative survival was calculated for each tumor line (parental and temozolomide resistant) as the ratio of median survival between temozolomide and placebo-treated mice and compared across all lines using a 2-sample rank-sum test. Differences in the in vitro cell survival, PCR band intensity, and pyrrosequencing were analyzed using a 2-sample t test.

Results

Establishment of pair-matched temozolomide-resistant and parental GBM lines

The Mayo GBM panel was established by implantation of patient samples into mice and maintained by serial heterotopic transplantation. Using this method, xenografts faithfully maintain the histopathologic and molecular characteristics of the primary patient tumor samples. Similar to clinical results, the benefit of temozolomide in orthotopically implanted xenografts correlated highly with MGMT promoter methylation and protein expression (20). From this panel, 4 temozolomide-sensitive tumors (GBM12, 14, 22, and 39) and 1 relatively resistant tumor (GBM28) were subjected to in vivo selection for temozolomide resistance. These derivative temozolomide-resistant lines are referred to with a “TMZ” suffix added to the parental tumor designation (i.e., GBM12TMZ is derived from parental GBM12).

The efficacy of temozolomide in each derivative line was evaluated in an orthotopic survival study and compared with published results for the corresponding parental lines (Fig. 1 and Supplementary Fig. S1). As summarized in the Supplementary Table S1, the survival benefit associated with temozolomide therapy is significantly greater in each of the parental lines as compared with the derivative temozolomide-resistant line (median relative survival for temozolomide treatment relative to placebo: parental lines 3.6 versus temozolomide resistant line 1.2; P < 0.01). Thus, the temozolomide selection method generated highly temozolomide-resistant tumor lines.

Reexpression of MGMT in temozolomide-resistant GBM xenografts

MGMT expression is an important mediator of temozolomide resistance (3, 13, 18, 25), and therefore, the MGMT protein and mRNA levels were evaluated in the paired lines. Consistent with epigenetic regulation of expression, MGMT protein was undetectable in the 3 parental MGMT-methylated lines (GBM12, 22, and 39) and in 1 partially methylated line (GBM28) but readily detectable in the unmethylated line (GBM28; Fig. 2A). In contrast, MGMT protein was significantly elevated in 2 derivative lines (GBM12TMZ and GBM14TMZ) and further elevated in the GBM28TMZ line. Similarly, MGMT mRNA was increased only in GBM12TMZ, GBM14TMZ, and GBM28TMZ (Fig. 2B). Thus, MGMT upregulation seems to be a common occurrence in the derivative temozolomide-resistant xenografts.

The contribution of MGMT to resistance was evaluated in vitro in GBM12TMZ, GBM14TMZ, and GBM22TMZ in a cytotoxicity assay using O6-BG, a selective MGMT inhibitor. While temozolomide was ineffective in all 3 resistant lines, cotreatment with O6-BG significantly enhanced
temozolomide sensitivity in GBM12TMZ and GBM14TMZ but not GBM22TMZ (Fig. 2C). Specifically, treatment with 30 μmol/L TMZ without or with O6-BG was associated with a relative absorbance, compared with control treatment, of 0.83 ± 0.08 and 0.25 ± 0.14, respectively (P = 0.012), for GBM12TMZ, 0.93 ± 0.16 and 0.18 ± 0.01, respectively (P = 0.016), for GBM14TMZ, and 0.84 ± 0.1 and 0.84 ± 0.17, respectively (P = 0.494), for GBM22TMZ. A neurosphere formation assay with GBM12TMZ and treatment with temozolomide plus O6-BG resulted in a significant reduction in neurosphere formation (14 ± 4.4) as compared with temozolomide alone (98 ± 3.8; P < 0.0001), whereas O6-BG alone had no impact on neurosphere formation (Fig. 2D). Together, these data show that the temozolomide resistance observed in GBM12TMZ and GBM14TMZ is mechanistically linked to overexpression of MGMT.

Delineation of epigenetic changes within MGMT promoter region

Because promoter methylation is an important mechanism regulating MGMT expression and temozolomide response in tumors (3), the paired lines were analyzed for methylation by MS-PCR. Despite marked upregulation of MGMT in GBM12TMZ and GBM14TMZ, MS-PCR comparison of parental and temozolomide-resistant lines showed no change in promoter methylation status for any tumor pair (Fig. 3A). To evaluate the methylation of the MGMT promoter in greater detail, pyrosequencing of the CpG sites (CpG 75–96; ref. 23) encompassing a region covered by the MS-PCR assay was conducted on GBM12, 14, 22, and the derivative temozolomide lines. As shown in Fig. 3B, the average percentage of methylation of all sites was 71.1% ± 15.2% for GBM12 versus 74.0% ± 19.6% for GBM12TMZ (P = 0.641), 28.4% ± 1.4% for GBM14 versus 24.0% ± 1.9% for GBM14TMZ (P = 0.139), and 73.2% ± 19.3% for GBM22 versus 80.8% ± 16.3% for GBM22TMZ (P = 0.211). In an analysis of methylation of each individual CpG, relatively subtle changes in the extent of CpG methylation were noted in a comparison between GBM12/12TMZ lines with statistically significantly increased methylation seen in 11 CpGs and decreased methylation seen in 4 CpGs. Similar comparison between GBM14/14TMZ lines revealed statistically significantly increased methylation in 2 CpGs and decreased methylation in 3 CpGs. In GBM22, only 2 CpGs showed elevated methylation, whereas no CpGs had decreased methylation (Supplementary Table S2).
Thus, there were no major changes in MGMT promoter methylation status that can explain the MGMT upregulation observed in GBM12TMZ and GBM14TMZ. Because posttranslational modifications of histones can modulate chromatin structure and control gene expression (26), acetylation and methylation of a key lysine residue, H3K9, and methylation of H3K27 was assessed by ChIP in paired flank tumor specimens from GBM12, 14, and 22. Consistent with a closed chromatin structure and low MGMT expression, all 3 parental tumors GBM12, 14, and 22 had low H3K9-ac and high H3K9-me2 occupancy within the MGMT promoter (Fig. 4A–C). In contrast, elevated MGMT expression in GBM12TMZ and GBM14TMZ was associated with a significant increase in H3K9-ac and concomitant decrease in H3K9-me2 promoter occupancy compared with parental tumors (Fig. 4A and B), whereas these changes were not seen in GBM22TMZ (Fig. 4C). No significant change in H3K27-me3 status was observed in any of the lines (Fig. 4A–C). The increased MGMT expression, mediated by chromatin changes in GBM12TMZ, was associated with recruitment of the transcription factors Sp1, C-JUN, NF-κB, and p300 to the MGMT promoter (Fig. 4D). Collectively, these data suggest that MGMT upregulation linked with the acquisition of temozolomide resistance is modulated by histone posttranslational modifications that favor transcriptionally active chromatin within the MGMT promoter.

Influence of HDAC inhibition on MGMT upregulation
Temozolomide resistance selection was repeated in GBM12 using multiple mice with established heterotopic xenografts and a clinically relevant dosing schedule. Twenty
mice with established tumors were treated with placebo or temozolomide for 5 days every 28 days for 3 cycles and then allowed to regrow. Once tumors exceeded 1,500 mm³, mice were euthanized and tumors were used to derive neurosphere cultures and archived for molecular analysis. As seen in Fig. 5A, only 2 of 10 temozolomide-treated xenografts harbored MGMT expression at the time of rechallenge (MGMT+). The absence of MGMT expression in the majority of tumors allowed for the identification of MGMT-mediated temozolomide resistance even within the same tumor.

Consistent with the initial findings in GBM12TMZ, only those lines with MGMT elevation showed increased H3K9-ac occupancy within the MGMT promoter region (Fig. 5C). One of the MGMT-expressing GBM12TMZ tumors (#0461) lost MGMT expression with subsequent passages in mice without treatment, but even after losing MGMT expression, the tumor remained resistant to temozolomide and resistance emergence was tested in a second GBM12 TMZ + O6-BG xenograft series (GBM12TMZ:MGMT–MGMT+:0461:MGMT−MGMT+) and non–MGMT-expressing tumors (MGMT−). Consistent with the initial findings in Figure 5D, bottom), consistent with persistent resistance in this subgroup, subsequent in vivo temozolomide rechallenge failed to delay the growth (data not shown) and resulted in partial restoration of MGMT expression. Thus, while the mechanism(s) of temozolomide resistance in the non–MGMT-expressing tumors is being evaluated, these data show the potential for heterogeneous mechanisms of temozolomide resistance development even within the same tumor.

Because the above findings suggest that MGMT-mediated temozolomide resistance is associated with increased histone acetylation, the influence of HDAC inhibition on resistance emergence was tested in a second GBM12...
temozolomide at recurrence, whereas 5 of the 8 evaluable tumors from temozolomide alone or SAHA alone showed MGMT upregulation. In this experiment, none of the tumors treated with temozolomide vs. temozolomide + SAHA showed MGMT upregulation, HDAC inhibitor therapy promoted an MGMT-mediated mechanism of temozolomide resistance in GBM12 xenografts.

Figure 6. Effect of SAHA on the evolution of acquired resistance to temozolomide. A, effect of SAHA on the H3K9 acetylation in pooled tissue samples from brain (B), liver (L), and the tumor (T) obtained from the correlative mice after 1 and 3 doses. B, the time to exceed 1,000 mm³ after treatment with placebo, temozolomide alone, SAHA alone, or temozolomide plus SAHA. C, evaluation of MGMT mRNA expression in recurrent xenografts following treatment with temozolomide alone (n = 10), SAHA alone (n = 10), and SAHA + temozolomide (n = 8) by qRT-PCR. The y-axis represents MGMT levels relative to the level in the placebo-treated tumors. D, short-term GBM12TMZ explant cultures derived from mice treated with temozolomide alone (0604T), temozolomide + SAHA (5485T + S and 5500T + S) were treated with graded concentrations of temozolomide with or without 10 μmol/L 6-BG and analyzed for neurosphere formation 14 days later. Results are the mean relative neurosphere number (± SEM), respectively, from 3 independent experiments. E, ChIP analysis of MGMT H3K9-ac among different treatment groups (PL, placebo; T, temozolomide; S, SAHA; T + S, temozolomide + SAHA). The MGMT expression level (MGMT status) for individual tumors is noted below the figure.

experiment. Similar as before, 40 mice with established xenografts were randomized to therapy with placebo, monotherapy with SAHA or temozolomide, or combination therapy. Additional mice were treated with SAHA alone for 1 or 3 doses and then euthanized to evaluate HDAC activity. As seen in Fig. 6A, global acetylation of H3K9 was markedly elevated in brain, liver, and flank tumor following drug therapy, compared with placebo-treated mice. In the tumor growth assay, there was no difference in the time for flank tumors to reach 1,000 mm³ in mice treated with placebo or SAHA (median survival 26 days vs. 31 days, respectively; P = 0.16), whereas both temozolomide treatment (median survival 75 days) and SAHA + temozolomide (median survival 92.5 days) significantly extended the time for tumor growth beyond 1,000 mm³ compared with placebo (P < 0.01; Fig. 6B and Supplementary Fig. S3A). Interestingly, despite no difference in treatment efficacy between temozolomide vs. temozolomide + SAHA (P = 0.12), SAHA cotreatment promoted MGMT upregulation. In this experiment, none of the tumors treated with temozolomide alone or SAHA alone showed MGMT upregulation at recurrence, whereas 5 of the 8 evaluable tumors from temozolomide + SAHA treatment group had high-level MGMT expression (Fig. 6C). Temozolomide sensitivity was restored with 6-BG cotreatment in the MGMT-expressing GBM12TMZ + SAHA tumor #5500 (relative neurosphere/well was 0.14 ± 0.02 as compared with 0.93 ± 0.01 for temozolomide alone, P < 0.01; Fig. 6D), whereas no such effect was observed in the non–MGMT-expressing GBM12TMZ tumor #0604 and non–MGMT-expressing GBM12TMZ + SAHA tumor #5485 [relative neurosphere/well was 0.88 ± 0.05 vs. 0.84 ± 0.03, (P > 0.05) and 0.85 ± 0.01 vs. 0.87 ± 0.03 (P > 0.05), respectively]. There were no significant differences in neurosphere formation efficiency among these resistant lines (Supplementary Fig. S3B). Consistent with a link between histone acetylation and MGMT upregulation, increased H3K9-ac was specifically observed in MGMT-expressing GBM12 xenografts (Fig. 6E), and ChIP assay revealed increased recruitment of SP1, C-JUN, NF-κB, and p300 to the MGMT promoter only in the MGMT-expressing GBM12TMZ + SAHA tumor #5500 (Supplementary Fig. S3C). Thus, consistent with a link between histone acetylation and MGMT upregulation, HDAC inhibitor therapy promoted an MGMT-mediated mechanism of temozolomide resistance in GBM12 xenografts.
Acquired Temozolomide Resistance in GBM

Discussion

The efficacy of temozolomide is limited by rapid emergence of resistance, and yet little is known about the underlying mechanisms of acquired resistance in the majority of GBM. Progress in this area has been slow due to the limited number of clinically relevant models for studying mechanisms of temozolomide resistance. The Mayo GBM xenograft panel is maintained exclusively by serial heterotopic transplantation in nude mice, and the MGMT promoter methylation status of primary patient tumor specimens is preserved in most xenografts that are subjected to multiple serial tumor passages. Moreover, response to temozolomide in this panel was significantly associated with MGMT promoter methylation status, and in a subset of tumors derived from patients ultimately treated with temozolomide, there was a good correlation between clinical response to temozolomide therapy and sensitivity of the corresponding xenografts to temozolomide (20, 23). Finally, it is possible that these xenograft GBM models are highly relevant to the heterogeneous mechanisms of temozolomide resistance. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide therapy. Collectively, these data suggest that the xenograft GBM models are highly relevant for analyzing mechanisms of temozolomide resistance.

MGMT repairs cytotoxic O6-MG DNA lesions induced by temozolomide. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide resistance, with suppression of MGMT activity resulting in increased cytotoxicity and MGMT overexpression resulting in resistance (7). However, the role of MGMT in the evolution of acquired temozolomide resistance is not well established. This is a major mechanism of resistance, and its potential role in the development of resistance may be driven by MGMT only in those cases where MGMT expression is maintained. The Mayo GBM xenograft panel is maintained exclusively by serial heterotopic transplantation in nude mice, and the MGMT promoter methylation status of primary patient tumor specimens is preserved in most xenografts that are subjected to multiple serial tumor passages. Moreover, response to temozolomide in this panel was significantly associated with MGMT promoter methylation status, and in a subset of tumors derived from patients ultimately treated with temozolomide, there was a good correlation between clinical response to temozolomide therapy and sensitivity of the corresponding xenografts to temozolomide (20, 23). Finally, it is possible that these xenograft GBM models are highly relevant to the heterogeneous mechanisms of temozolomide resistance. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide therapy. Collectively, these data suggest that the xenograft GBM models are highly relevant for analyzing mechanisms of temozolomide resistance.

MGMT repairs cytotoxic O6-MG DNA lesions induced by temozolomide. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide resistance, with suppression of MGMT activity resulting in increased cytotoxicity and MGMT overexpression resulting in resistance (7). However, the role of MGMT in the evolution of acquired temozolomide resistance is not well established. This is a major mechanism of resistance, and its potential role in the development of resistance may be driven by MGMT only in those cases where MGMT expression is maintained. The Mayo GBM xenograft panel is maintained exclusively by serial heterotopic transplantation in nude mice, and the MGMT promoter methylation status of primary patient tumor specimens is preserved in most xenografts that are subjected to multiple serial tumor passages. Moreover, response to temozolomide in this panel was significantly associated with MGMT promoter methylation status, and in a subset of tumors derived from patients ultimately treated with temozolomide, there was a good correlation between clinical response to temozolomide therapy and sensitivity of the corresponding xenografts to temozolomide (20, 23). Finally, it is possible that these xenograft GBM models are highly relevant to the heterogeneous mechanisms of temozolomide resistance. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide therapy. Collectively, these data suggest that the xenograft GBM models are highly relevant for analyzing mechanisms of temozolomide resistance.

MGMT repairs cytotoxic O6-MG DNA lesions induced by temozolomide. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide resistance, with suppression of MGMT activity resulting in increased cytotoxicity and MGMT overexpression resulting in resistance (7). However, the role of MGMT in the evolution of acquired temozolomide resistance is not well established. This is a major mechanism of resistance, and its potential role in the development of resistance may be driven by MGMT only in those cases where MGMT expression is maintained. The Mayo GBM xenograft panel is maintained exclusively by serial heterotopic transplantation in nude mice, and the MGMT promoter methylation status of primary patient tumor specimens is preserved in most xenografts that are subjected to multiple serial tumor passages. Moreover, response to temozolomide in this panel was significantly associated with MGMT promoter methylation status, and in a subset of tumors derived from patients ultimately treated with temozolomide, there was a good correlation between clinical response to temozolomide therapy and sensitivity of the corresponding xenografts to temozolomide (20, 23). Finally, it is possible that these xenograft GBM models are highly relevant to the heterogeneous mechanisms of temozolomide resistance. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide therapy. Collectively, these data suggest that the xenograft GBM models are highly relevant for analyzing mechanisms of temozolomide resistance.

MGMT repairs cytotoxic O6-MG DNA lesions induced by temozolomide. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide resistance, with suppression of MGMT activity resulting in increased cytotoxicity and MGMT overexpression resulting in resistance (7). However, the role of MGMT in the evolution of acquired temozolomide resistance is not well established. This is a major mechanism of resistance, and its potential role in the development of resistance may be driven by MGMT only in those cases where MGMT expression is maintained. The Mayo GBM xenograft panel is maintained exclusively by serial heterotopic transplantation in nude mice, and the MGMT promoter methylation status of primary patient tumor specimens is preserved in most xenografts that are subjected to multiple serial tumor passages. Moreover, response to temozolomide in this panel was significantly associated with MGMT promoter methylation status, and in a subset of tumors derived from patients ultimately treated with temozolomide, there was a good correlation between clinical response to temozolomide therapy and sensitivity of the corresponding xenografts to temozolomide (20, 23). Finally, it is possible that these xenograft GBM models are highly relevant to the heterogeneous mechanisms of temozolomide resistance. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide therapy. Collectively, these data suggest that the xenograft GBM models are highly relevant for analyzing mechanisms of temozolomide resistance.

MGMT repairs cytotoxic O6-MG DNA lesions induced by temozolomide. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide resistance, with suppression of MGMT activity resulting in increased cytotoxicity and MGMT overexpression resulting in resistance (7). However, the role of MGMT in the evolution of acquired temozolomide resistance is not well established. This is a major mechanism of resistance, and its potential role in the development of resistance may be driven by MGMT only in those cases where MGMT expression is maintained. The Mayo GBM xenograft panel is maintained exclusively by serial heterotopic transplantation in nude mice, and the MGMT promoter methylation status of primary patient tumor specimens is preserved in most xenografts that are subjected to multiple serial tumor passages. Moreover, response to temozolomide in this panel was significantly associated with MGMT promoter methylation status, and in a subset of tumors derived from patients ultimately treated with temozolomide, there was a good correlation between clinical response to temozolomide therapy and sensitivity of the corresponding xenografts to temozolomide (20, 23). Finally, it is possible that these xenograft GBM models are highly relevant to the heterogeneous mechanisms of temozolomide resistance. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide therapy. Collectively, these data suggest that the xenograft GBM models are highly relevant for analyzing mechanisms of temozolomide resistance.
MGMT overexpression likely is an important mechanism of resistance in both MGMT promoter hypermethylated and unmethylated GBM tumors. In this study, MGMT overexpression was regulated epigenetically and was specifically potentiated by concurrent HDAC inhibition by SAHA (Fig. 6). While H3K9 acetylation was globally increased by SAHA treatment (Fig. 6A), this mark was only enriched within MGMT promoter of the MGMT-expressing GBM12TMZ lines derived from the combined temozolomide–SAHA treatment (Fig. 6E). This finding contradicts recent publications, in which SAHA alone induced H3K9-ac mediated reactivation of MGMT expression in promoter hypermethylated leukemic and breast cancer cells in vitro (31). This difference in results may stem from the use of primary xenograft models compared with established cell lines and the exclusive analysis of SAHA effects in vivo in which metabolism of the drug will lead to fluctuating exposure over time compared with relatively steady concentrations of SAHA encountered in vitro. Moreover, as temozolomide treatment alone can induce heterochromatin reorganization (32), we speculate that temozolomide-induced epigenetic events may contribute to heterochromatin remodeling within the MGMT promoter associated with cotreatment with SAHA.

Because there is an ongoing clinical trial evaluating the integration of SAHA with temozolomide and radiotherapy in newly diagnosed patients with GBM, the results in this study are reassuring that concurrent SAHA did not accelerate the development of temozolomide resistance, although concurrent therapy resulted in a distinct shift favoring MGMT upregulation. To our knowledge, this is the first ever demonstration that HDAC inhibition can influence the mechanism of resistance emergence when combined with a conventional cytotoxic chemotherapy agent. With an increasing number of epigenetically targeted therapies being tested in oncology, these results highlight the potential for these novel therapies to affect resistance emergence.

Disclosure of Potential Conflicts of Interest
J.N. Sarkaria has a commercial research grant from Merck, Basilea, and Genentech. No potential conflicts of interest were disclosed by other authors.

Authors’ Contributions
Conception and design: G.J. Kitange, R. Urrutia, J.N. Sarkaria

Development of methodology: G.J. Kitange, B.L. Carlson, M.A. Schroeder, L. Cen, J.N. Sarkaria

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G.J. Kitange, A.C. Mladek, B.L. Carlson, M.A. Schroeder, J.L. Pokorny, L. Cen, S.K. Gupta

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G.J. Kitange, B.L. Carlson, P.A. Decker, W. Wu, G. Lomberk, S.K. Gupta, R. Urrutia, J.N. Sarkaria

Writing, review, and/or revision of the manuscript: G.J. Kitange, P.A. Decker, R. Urrutia, J.N. Sarkaria

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.L. Carlson, H. Pokorny, G. Lomberk

Study supervision: R. Urrutia, J.N. Sarkaria

Acknowledgments
The authors thank Drs. Zhiguo Zhang and Martin Fernandez-Zapico for critical reading of the manuscript.

Grant Support
This work was supported by the Mayo Clinic and grants from the NIH: RO1 CA127716, RO1 CA141121, and the Mayo Brain Tumor SPORE P50 CA108361.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 16, 2012; revised May 15, 2012; accepted May 25, 2012; published OnlineFirst June 6, 2012.

References


Inhibition of Histone Deacetylation Potentiates the Evolution of Acquired Temozolomide Resistance Linked to MGMT Upregulation in Glioblastoma Xenografts


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-0560

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/06/06/1078-0432.CCR-12-0560.DC1

Cited articles
This article cites 32 articles, 14 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/15/4070.full.html#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
/content/18/15/4070.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.