Distinct molecular mechanisms of acquired resistance to temozolomide in glioblastoma cells

Happold, C ; Roth, P ; Wick, W ; Schmidt, N ; Florea, A M ; Silginer, M ; Reifenberger, G ; Weller, M

Abstract: J. Neurochem. (2012) 10.1111/j.1471-4159.2012.07781.x ABSTRACT: Temozolomide (TMZ) is an alkylating chemotherapeutic agent that prolongs the survival of patients with glioblastoma. Clinical benefit is more prominent in patients with methylation of the O(6) -methyl-guanine DNA methyltransferase (MGMT) promoter. However, all patients eventually suffer from tumor progression because their tumors become resistant to TMZ. Here, we modeled acquired TMZ resistance in glioma cells in vitro to identify underlying molecular mechanisms. To this end, the glioma cell lines LNT-229, LN-308, and LN-18 were exposed repetitively to increasing concentrations of TMZ to induce a stable resistant phenotype (R) defined by clonogenic survival assays. The molecular mechanisms mediating acquired resistance were assessed by immunoblot, PCR, and flow cytometry. Rescue experiments were performed with siRNA-mediated candidate gene silencing. We found in LN-18 cells constitutively expressing MGMT a strong up-regulation of MGMT levels in TMZ-resistant cells. TMZ resistance in the MGMT-negative cell lines LNT-229 and LN-308 was not associated with de novo expression of MGMT. Instead, we found a down-regulation of several DNA mismatch-repair proteins in resistant LNT-229 cells. A TMZ-resistant phenotype was also achieved by silencing selected DNA mismatch repair proteins in parental LNT-229 cells. No obvious mechanism of resistance was identified in the third cell line, LN-308, except for reduced methylation of LINE-1 repetitive elements. In conclusion, we demonstrate that different molecular mechanisms may contribute to the development of acquired TMZ resistance in glioma cells, indicating the need to develop distinct strategies to overcome resistance.

DOI: https://doi.org/10.1111/j.1471-4159.2012.07781.x

Distinct molecular mechanisms of acquired resistance to temozolomide in glioblastoma cells

Caroline Happold[1]*, Patrick Roth[1], Wolfgang Wick[2], Natalie Schmidt[3], Ana-Maria Florea[3], Manuela Silginer[1], Guido Reifenberger[3], and Michael Weller[1]

[1]Laboratory of Molecular Neuro-Oncology, Department of Neurology, University Hospital Zurich, Switzerland, [2]Department of Neuro-Oncology, Neurology Clinic and National Center for Tumor Disease, Heidelberg, Germany [3]Department of Neuropathology, University Hospital Düsseldorf, Germany

***Correspondence:** Caroline Happold, M.D., Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 26, 8091 Zurich, Switzerland, Tel.: +41 44 255 1111, Fax: +41 (0)44 255 4380, E-mail: caroline.happold@usz.ch

Running title: Acquired temozolomide resistance in glioblastoma

Key words: glioma, temozolomide, mismatch repair, chemoresistance, MGMT

ABSTRACT

Temozolomide (TMZ) is an alkylating chemotherapeutic agent that prolongs the survival of patients with glioblastoma. Clinical benefit is more prominent in patients with methylation of the O^6-methyl-guanine DNA methyltransferase (*MGMT*) promoter. However, all patients eventually suffer from tumor progression because their tumors become resistant to TMZ. Here, we modelled acquired TMZ resistance in glioma cells *in vitro* to identify underlying molecular mechanisms. To this end, the glioma cell lines LNT-229, LN-308 and LN-18 were exposed repetitively to increasing concentrations of TMZ to induce a stable resistant phenotype (R) defined by clonogenic survival assays. The molecular mechanisms mediating acquired resistance were assessed by immunoblot, PCR and flow cytometry. Rescue experiments were performed with siRNA-mediated candidate gene silencing. We found in LN-18 cells constitutively expressing MGMT a strong up-regulation of MGMT levels in TMZ-resistant cells. TMZ resistance in the MGMT-negative cell lines LNT-229 and LN-308 was not associated with *de novo* expression of MGMT. Instead, we found a down-regulation of several DNA mismatch-repair proteins in resistant LNT-229 cells. A TMZ-resistant phenotype was also achieved by silencing selected DNA mismatch repair proteins in parental LNT-229 cells. No obvious mechanism of resistance was identified in the third cell line, LN-308, except for reduced methylation of LINE-1 repetitive elements. In conclusion, we demonstrate that different molecular mechanisms may contribute to the development of acquired TMZ resistance in glioma cells, indicating the need to develop distinct strategies to overcome resistance.

Abbreviations

Anx, annexin; APNG, alkylpurine-N-glykosylase; ATM, Ataxia Telangiectasia Mutated protein; ATR, Ataxia Telangiectasia and Rad3-related protein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ECL, enhanced chemoluminescence; FCS, fetal calf serum; fwd, forward; HRP, horseradish peroxidase; MGMT, O^6-methyl-guanine DNA methyltransferase; MLH1, MutL homolog 1; MMR, mismatch repair; MSH, MutS homolog; MSP, methylation-specific polymerase chain reaction; O^6-BG: O^6-benzylguanine; PI, propidium iodide; PMS1, postmeiotic segregation increased, S. cerevisiae 1; rv, reverse; TMZ, temozolomide; TSA, trichostatin A.

INTRODUCTION

Despite recent therapeutic advances, the prognosis of patients afflicted by glioblastoma remains poor, with a progression-free survival in the range of months, even with multimodal therapy including surgery, radio- and chemotherapy. Temozolomide (TMZ), an oral alkylating agent, has demonstrated activity against recurrent and newly diagnosed glioblastoma and is being used as the standard of care since 2000 respectively 2005 (Yung *et al.* 2000, Stupp *et al.* 2005).

The DNA repair protein O^6-methylguanine methyltransferase (MGMT) removes O^6-alkyl-guanine adducts from DNA through irreversible binding and degradation, thereby minimizing the DNA-damaging effects of alkylating agent chemotherapy (Wang *et al.* 1996, Phillips *et al.* 1997). Accordingly, pharmacological inhibition of MGMT promotes antitumor activity of alkylating agents such as TMZ both *in vitro* and *in vivo* (Dolan *et al.* 1991, Wedge *et al.* 1996). Epigenetic silencing of the *MGMT* gene in tumor cells by methylation of its promoter had been associated with glioma sensitivity to alkylating chemotherapy (Esteller *et al.* 2000) and has been identified as a predictive biomarker for benefit from TMZ in patients with newly diagnosed glioblastoma (Hegi *et al.* 2005, Weller *et al.* 2010).

However, even patients with initial tumor control will inevitably relapse or progress during or after TMZ therapy. Thus, both constitutive and acquired glioma cell resistance to alkylating chemotherapy are major clinical challenges. A better understanding of the underlying mechanisms may allow for a more durable benefit from the anti-glioma properties of TMZ.

In contrast to its role in primary chemoresistance, the role of MGMT in acquired resistance to TMZ has only partially been assessed. Few, if any, patients exhibit a

change of MGMT promoter methylation status at progression (Brandes *et al.* 2010, Christmann *et al.* 2010, Felsberg *et al.* 2011).

Another DNA repair system that has been proposed to mediate chemoresistance in various tumor cells is the DNA mismatch repair (MMR) system, which consists of several enzymes. The role of deficient MMR has been addressed in most detail in colon cancer. Here, MMR mutations cause genetic predisposition associated with an increased risk for colon cancer (Bronner *et al.* 1994, Papadopoulos *et al.* 1994). The MMR system recognizes and repairs base mismatches after DNA replication, mostly mediated by two heterodimers of MutS homologues, MutS-α (MSH2/MSH6 heterodimer) for small insertion/deletion loops and MutS-β (MSH2/MSH3 heterodimer) for large insertion/deletion loops (Genschel *et al.* 1998). Colon carcinoma cells are up to 60x more resistant to alkylating agent chemotherapy when the MMR genes are mutated (Liu *et al.* 1996).

In human gliomas, the expression of 6 MMR proteins has been detected. Their role has not been well elucidated yet. While some reports postulate that an overexpression of MMR proteins exerts oncogenic effects, most probably by continuous DNA repair promoting survival (Rellecke *et al.* 2004), other authors have shown that a defective MMR system may favor tumor growth and chemoresistance. It is assumed that a lack of correct DNA replication leads to an accumulation of DNA damage and subsequent tumor growth (Yip *et al.* 2009).

MATERIALS AND METHODS

Materials and cell lines

All glioma cell lines were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland). TMZ-resistant cells were generated by repetitive pulse exposure of LNT-229, LN-308 and LN-18 glioma cells to TMZ (24 h exposure every 2 weeks). Increasing concentrations of TMZ were applied over 6 months under consideration of the respective EC_{50} values (Hermisson *et al.* 2006), with selection and culturing of the resistant surviving cell fraction of each passage and consecutive re-exposure to a TMZ pulse after confluent growth. The derived resistant cell lines are further referred to as LNT-229_R, LN-308_R and LN-18_R. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 2 mM glutamine and penicillin (100 IU/ml)/streptomycin (100 μg/ml). TMZ was provided by Schering-Plough (Kenilworth, NJ, USA). A stock solution of TMZ at 50 mM was prepared in dimethylsulfoxide (DMSO) and stored at -20°C. Propidium iodide (PI), trichostatin A and all other reagents, unless indicated otherwise, were purchased from Sigma (St. Louis, MO, USA).

Viability and clonogenic assays

Proliferation was assessed by obtaining viable cell counts using trypan blue dye exclusion. For cytotoxicity assays, 5 x 10^3 cells were seeded per well in 96-well plates, allowed to attach for 24 h, and exposed to TMZ as indicated for 72 h in serum-free medium. Clonogenic assays were performed by seeding 10^2 cells for LNT-229 and LN-18 or 3 x 10^2 cells for LN-308 per well in 96-well plates. After 24 h, the cells were exposed to TMZ as indicated for 24 h in serum-free medium, followed

by observation for 7-14 days in FCS-containing medium. For both assays, cell density was assessed by crystal violet staining of adherent cells.

Immunoblot analyses

The cells were treated with TMZ or DSMO vehicle and lysed in lysis buffer containing 2 µg/ml Aprotinin, 10 µg/ml Leupeptin and 100 µg/ml PMSF (Sigma). Proteins (20 µg/lane) were separated on 8-12% acrylamide gels. After transfer to nitrocellulose (Biorad, Munich, Germany), the blots were blocked in PBS containing 5% skim milk and 0.05% Tween 20 and incubated overnight at 4°C with primary antibodies and 1 h at room temperature for secondary antibodies. Visualization of protein bands was accomplished using horseradish peroxidase (HRP)-coupled secondary antibodies (Santa Cruz / Healthcare) and enhanced chemiluminescence (Pierce / Thermo Fisher, Madison, WI, USA) using a G:BOX imager (Syngene, Cambridge, UK). The following antibodies were used: anti-MGMT from Alpha Diagnostics (San Antonio, TX, USA), anti-p21 from BD Bioscience (Heidelberg, Germany), antibodies to p53 MSH3 and alkylpurine-N-glykosylase (APNG) from Santa Cruz (Santa Cruz, CA, USA), PMS2, MSH6 and MLH1 antibodies from BD (Franklin Lakes, NJ, USA), antibody to MSH2 from Calbiochem/Merck (Darmstadt, Germany), anti-PMS1 from Abcam (Cambridge, UK), and anti-GAPDH from Everest Biotech (Oxfordshire, UK).

For immunoblot analysis of histone modificatons, the cells were lysed in 250 µl cell lysis buffer (50 mM Tris HCL, pH=8, 150 mM NaCl, 0,5% Triton X-100, 0.5% deoxycholate) containing 1 x protease inhibitors (Roche GmbH, Ref.: 1183617001). Proteins (30 µg/lane) were separated on 15% acrylamide gels (Tricin-SDS PAGE). After transfer to nitrocellulose membranes (Whatman GmbH, Dassel), the blots were blocked in TBS containing 5% milk and 0.1% Tween 20 and incubated overnight at 4°C with primary antibodies and 1 h at room temperature with horseradish

peroxidase (HRP)-coupled secondary antibodies (Pierce Technology, Rockford, IL; Thermo Fisher Scientific, Waltham, MA). Visualization of protein bands was performed by using Immobilon^{TM} Western HRP Substrate containing luminol and peroxide solution (Millipore, Billerica, MA). Chemiluminescence was recorded with the LAS-3000 mini imager (FUJIFILM Life Science, Stanford, CT). The following primary antibodies were used: anti-H3 (# 17-10046, 1:1000), anti-H3ac (# 06-599, 1:10000) and anti-H3K9me3 (# 17-625, 1:1000) from Upstate (Charlotteville, VA), and anti-tubulin (# T9026, 1:3000) from Sigma-Aldrich GmbH (Steinheim, Germany)

Quantitative reverse transcriptase PCR

Total RNA from untreated cells or cells exposed to the respective agents, TMZ for 24 h, TSA for 48 h, was prepared using the NucleoSpin System (Macherey-Nagel AG, Oensingen SO, Switzerland) and transcribed into cDNA according to standard protocols using random primer (Bioconcept/NEB, Bioconcept, Allschwil, Switzerland) and Superscript II reverse transcriptase (Invitrogen, CA, USA). For real-time PCR, cDNA amplification was monitored using SYBRGreen chemistry on the 7300 Real time PCR System (Applied Biosystems, Zug, Switzerland). The conditions for these PCR reactions were: 40 cycles, 95°C/15 sec, 60°C/1 min, using the following specific primers: *GAPDH* forward (fwd): 5'-CTCTCTGCTCCTCCTGTTCGAC-3', *GAPDH* reverse (rv): 5'-TGAGCGATGTGGCTCGGCT-3'; *MGMT* fwd: 5'-CAC TTC ACC ATC CCG TTT TCC-3', *MGMT* rv: 5'-TGC TGG TAA GAA ATC ACT TCT CC-3'; p21 fwd: 5'-GAC CAG CAT GAC AGA TTT CTA CCA-3', p21 rv: 5'-TTC CTG TGG GCG GAT TAG G-3', *MSH2* fwd: 5'-GCT GGA AAT AAG GCA TCC AAG G-3', *MSH2* rv: 5'-CAC CAA TGG AAG CTG ACA TAT CA-3'; *MSH3* fwd: 5'-TGG AAA ATG ATG GGC CTG TTA AA-3', *MSH3* rv: 5'-AGA CAT TCC CAG ATC ACT TCC T-3'; *MSH6* fwd: 5'-AGC TTA AAG GAT CAC GCC ATC-3', *MSH6* rv: 5'-AAG CAC ACA ATA GGC

TTT GCC-3'; *PMS1* fwd: 5'-GTT CTG GGG ACT GCT GTT ATG-3', *PMS1* rv: 5'-GGT CTG CAT CAC ACT TTG GAA-3'; *PMS2* fwd: 5'-GAA GGT TGG AAC TCG ACT GAT G-3', *PMS2* rv: 5'-CGC ACA GGT AGT GTG GAA AA-3'; *MLH1* fwd: 5'-TTC GTG GCA GGG GTT ATT CG-3', *MLH1* rv: 5'-GCC TCC CTC TTT AAC AAT CAC TT-3'; *ATM* fwd: 5'-TTC CAT ACC TGA AGT GTA GCA TAA A-3'; *ATM* rv: 5'-AAT TTG CCA GTC TCA TTA ACC C-3'; *ATR* fwd: 5'- TCT CAG CCA ACC TCC GTG AT-3'; *ATR* rv: 5'- GCA GAA GTC TCG TTA TGA TCC AA-3'; *cyclin D1* fwd: 5'-GAA CAA ACA GAT CAT CCG CAA AC-3'; *cyclin D1* rv: 5'-GCG GTA GTA GGA CAG GAA GTT G-3' *; p27* fwd: 5'-TAA TTG GGG CTC CGG CTA ACT-3'; *p27* rv: 5'-TTG CAG GTC GCT TCC TTA TTC-3'; APNG *fwd*: 5´- CCC ATA CCG CAG CAT CTA TT-3'; APNG *rv*: 5´- AGG TAT GCC TCG GTC TCC AC-3'. Data analysis was done using the $\Delta\Delta C_T$ method for relative quantification.

DNA mutation and methylation analyses

For methylation-specific PCR (MSP) analysis of the *MGMT* promoter, 2 pairs of primers, each specific for either the methylated or the unmethylated promoter region, were used. Primer sequences for the unmethylated sequence were fwd: 5′-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3′ and rv: 5′-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3′, and for the methylated sequence fwd: 5′-TTT CGA CGT TCG TAG GTT TTC GC-3′ and rv: 5′-GCA CTC TTC CGA AAA CGA AAC G-3′. The MSP reactions were prepared in a total volume of 25 µl using HotStarTaq® Master Mix Kit (Qiagen, Hilden, Germany). For PCR, 1.8 µl of bisulfite-modified DNA were added and subjected to 36 PCR cycles with denaturation at 94°C for 30 sec, primer annealing at 59°C for 40 sec, extension at 72°C for 2 min and a final extension for 10 min. PCR products were separated using Spreadex® EL 600 or 800 gels (Elchrom

Scientific AG, Cham, Switzerland) and loaded with 3–5 µl of the respective PCR product. Electrophoresis was carried out at 120 V and 55°C for 40 min. Amplicons were visualized using SYBR® Gold (Molecular Probes, OR) (Felsberg *et al.* 2009). In addition, MGMT promoter methylation was assessed by DNA pyrosequencing as reported (Felsberg et al. 2011).

Methylation analyses of the MLH1, MSH2, MSH6 and PMS2 gene promoters were assessed by DNA pyrosequencing as described (Felsberg et al. 2011). In addition, we investigated exons 1 to 10 of MSH6 for mutation using single-strand conformation polymorphism (SSCP) analysis followed by direct sequencing (Reifenberger *et al.* 1996). The respective primer sequences are available on request. The methylation level of LINE-1 (GenBank accession number X58075) was also assessed by DNA pyrosequencing using the PyroMark LINE-1 kit (Qiagen, Hilden, Germany) and the PyroMark Q24 sequencer (Qiagen).

Reporter assay

Dual luciferase/renilla assays were carried out with co-transfection of 150 ng of the pGL2-Luc MGMT (575/+24ML) reporter construct and 20 ng of the renilla reniformis-CMV (pRL-CMV) control plasmid (Promega, Madison, WI, USA). Luciferase activity was normalized to constitutive renilla activity. The pGL2-Luc MGMT (575/+24ML) construct (Biswas et al. 1999) was a kind gift from Dr. S. Mitra (Sealy Center for Molecular Science and Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX, USA).

Flow cytometry

For cell cycle analysis, LNT-229, LN-308 and LN-18 parental or resistant (R) cells were treated with TMZ or transfected with siRNA 24 h before analysis as indicated

below, harvested, fixed and permeabilized overnight in ice-cold 70% ethanol (Fluka /Sigma Aldrich Buchs, Switzerland). After washing with PBS, RNA was digested with RNase A (Carl Roth AG, Karlsruhe, Germany). DNA was stained with PI (50 μg/ml) and fluorescence was recorded in a CyAn ADP S2536 analyzer (Dako Cytomation, Carpinteria, CA). For analysis of cell death, cells were grown in six-well plates, incubated with TMZ at different concentrations for 24 h, washed in PBS and allowed to grow for 72 h. Annexin (Anx) V-fluorescein isothiocyanate (1:100) and PI (50 μg/ml) were added, and fluorescence in a total of 10,000 events per condition was recorded in a CyAn flow cytometer. AnxV-fluorescein isothiocyanate- or PI-positive cells were counted as dead cells, and the remaining cells were designated the surviving cell fraction.

RNA interference-mediated gene silencing

For transient transfections, 2.5×10^5 glioma cells were seeded in a six-well plate and exposed to 100 nmol/L of either specific or scrambled control small interfering (si)RNA, using Metafectene Transfection reagent (Biontex Laboratories GmbH, Martinsried, Germany) for 12 h. siRNA was purchased from Microsynth AG (Balgach, Switzerland). Cell pellets for FACS analysis were collected 24 h after transfection; RNA samples were collected 48 h after the first exposure to the siRNA; protein samples were collected 72 h post transfection. The following sequences were used: MSH2: 5'-GUC CAA GGU GAA ACA AAU GTT-3'; MSH3: 5'-CCA AAU AAC UAG AGG AAU UTT-3'; MSH6: 5'-CCU GAA AUA CUG AGA GCA ATT-3'; PMS1: 5'-GCG AAU GGU UUC AAG AUA ATT-3'; PMS2: 5'-CAA UGU UAC UCC AGA UAA ATT-3'; MLH1: 5'-GGA AGA UUC UGA UGU GGA ATT-3'; p21: 5'-GGA CAG CAG AGG AAG ACC ATT-3'.

Data analysis

Data are representative of experiments performed three times with similar results. Viability and proliferation studies were performed using triplicate wells. Where indicated, analysis of significance was performed using the two-tailed Student's t-test.

RESULTS

Repeated exposure to TMZ induces resistance in human glioma cells

LNT-229, LN-308 and LN-18 glioma cells were pulse-exposed to increasing concentrations of TMZ for 6 months until a stable resistant phenotype was observed. No change in cellular morphology was observed by light microscopy (Supplementary Figure 1). All resistant sublines (R) showed reduced proliferation compared with the parental lines although their baseline cell cycle distribution was unaltered. No differences between the cell lines could be identified in Annexin/PI-FACS, either (Supplementary Figure 2). Acute growth inhibition and clonogenic survival assays were used to characterize the sensitivity of these cells to TMZ. Parental cells without prior exposure to TMZ or the TMZ-pre-exposed glioma cells were exposed to increasing concentrations of TMZ. We noticed an up to 10-fold increase of the EC_{50} values in the resistant sublines in clonogenic assays (Figure 1A). In both paradigms, TMZ led to a concentration-dependent decrease of viable cell counts in all cell lines examined. LNT-229_R, LN-308_R and LN-18_R cells displayed reduced sensitivity to TMZ compared with parental cells in acute survival (Figure 1B) and clonogenic assays (Figure 1C). All resistant cell lines exhibited resistance features for at least more than 1 year after the last exposure to TMZ (Supplementary Figure 3). This induction of resistance was restricted to TMZ in LNT-229_R or LN-308_R cell lines that showed no cross-resistance to lomustine (CCNU), while there was cross-resistance in LN-18_R cells. No cross-resistance was observed for cytarabine in any cell line (Supplementary Figure 4). All further experiments were conducted under conditions mimicking the clonogenic cell death assays because clinically relevant concentrations of TMZ, which are in the range of 50-70 μM, are active in these

assays only, consistent with the observation that MGMT mediates resistance to TMZ only in these assays (Hermisson et al. 2006).

Up-regulation of MGMT contributes to acquired TMZ resistance in LN-18_R cells

The expression of MGMT predicts the sensitivity of the glioma cell lines used here to TMZ: in contrast to LNT-229 and LN-308 glioma cells, which exhibit strong *MGMT* promoter methylation with more than 90% of methylated alleles revealed by DNA pyrosequencing, LN-18 cells lack *MGMT* promoter methylation, constitutively express MGMT and are the most resistant cells examined here (Hermisson et al. 2006) (Figure 1A). No change in the methylation status was observed in the resistant LNT-229_R or LN-308_R cells compared with the respective parental cells (Figure 2A). Accordingly, no MGMT protein was demonstrated by immunoblotting in LNT-229_R and LN-308_R cells. In contrast, real-time PCR and immunoblotting revealed a strong increase in MGMT protein, mRNA and transcriptional activity levels in LN-18_R cells compared with LN-18 parental cells (Figure 2A).

MGMT is consumed in response to DNA damage by proteasomal degradation after removal of an alkyl group from the DNA (Srivenugopal *et al.* 1996). Both parental LN-18 and LN-18_R cells displayed a concentration-dependent loss of MGMT protein levels after exposure to TMZ. However, LN-18_R cells still retained MGMT levels after exposure to TMZ at 1000 µM that were similar to untreated parental cells (Figure 2B).

We next asked whether increased MGMT expression was responsible for induced TMZ resistance in LN-18_R cells. To this end, the cells were pretreated with the MGMT-depleting agent O^6-BG prior to the TMZ challenge. An exposure to O^6-BG for 2 h before administration of TMZ depleted MGMT from both LN-18 and LN-18_R

cells (Figure 2C) and largely restored TMZ sensitivity of LN-18_R cells in clonogenic survival assays (Figure 2D). The EC_{50} shift induced by O^6-BG was 3.1 in parental cells and 6.4 in resistant cells and thus more prominent in LN-18_R cells, corresponding to the contribution of increased MGMT levels to acquired resistance in this cell line. Thus, up-regulation of MGMT expression was identified as a mediator of resistance in one of the three cell lines examined here.

TMZ-resistant glioma cells display differential changes in cell cycle progression in response to TMZ

In order to further elucidate mechanisms underlying acquired TMZ resistance, we investigated cell cycle progression and the expression of cell cycle-related proteins in response to TMZ. In LNT-229 and LN-308 parental cells, exposure to TMZ induced a G2/M arrest in a concentration-dependent manner. In contrast, the resistant lines did not display any relevant change in cell cycle distribution. In parental LN-18 cells, TMZ induced acute cell death in a concentration-dependent manner, a finding that was much less prominent in LN-18_R cells. In contrast, the resistant LN-18 cells accumulated in G2/M (Figure 3A). This observation was confirmed by anxV/PI stainings which demonstrated an increase of the apoptotic fraction in LN-18 cells in response to TMZ that decreased to less than half in LN-18_R cells (Figure 3B). Thus, G2/M arrest was associated with prevention of growth in LNT-229 and LN-308 cells, but linked to clonogenic survival in LN-18 cells.

p21 displays altered expression in resistant LN-18_R cells

p21 is one of the major regulators of the cell cycle and has previously been linked to resistance to apoptosis in glioma cells (Naumann *et al.* 1998, Glaser *et al.* 2001). Real-time PCR and immunoblot revealed elevated p21 mRNA and protein levels in

untreated LN-18_R, but not in LNT-229_R or LN-308_R cells (Figure 4A, B). Moreover, exposure to TMZ strongly up-regulated p21 mRNA levels in LN-18_R cells (Figure 4C), but not in LNT-229_R or LN-308_R (data not shown). Accordingly, the doubling time in LN-18_R cells was distinctively reduced (Supplementary Figure 2). In contrast to p21, no significant changes were observed for ATM, ATR, p27 or cyclin D in either cell line (Supplementary Figure 5). Since LN-18 cells express a mutant p53 protein (Wischhusen *et al.* 2003), the induction of p21 expression is independent of p53. We also considered the possibility that the increased p21 levels were responsible for slowed cell cycle progression in LN-18-R cells. Accordingly, we silenced p21 expression in LN-18 or LN-18-R cells using siRNA. Interestingly, the main effect of silencing p21 was the acute induction of cell death rather than an altered cell cycle distribution, and the extent of cell death induction was more prominent in LN-18-R cells which expressed more p21 (37.5% versus 24.2%) (Supplementary Figure 6). Thus, a role for p21 specifically in cell cycle progression could not be confirmed.

MMR protein down-regulation is important for TMZ-resistance in LNT-229-R

Reduced levels of MMR proteins may promote resistance to alkylating agents, but their role in gliomas has remained controversial. While no significant changes in the mRNA expression and protein levels of MLH1, MSH2, MSH3, MSH6, PMS1 and PMS2 were observed in LN-308_R and LN-18_R cells (Supplementary Figure 7), real-time PCR and immunoblot analysis revealed reduced expression of all MMR molecules in LNT-229-R cells (Figure 5A, B). Reduced MMR protein levels were not a consequence of aberrant promoter methylation of MLH1, MSH2, MSH6 and PMS2 as assessed by DNA pyrosequencing (data not shown). SSCP analysis of exons 1 to 10 of MSH6 revealed no sequence differences in LN-18_R and LNT-229_R

compared with their respective parental cells. LN-308_R demonstrated an intronic point mutation c.412+5C>T which was not detected in the parental LN-308 cells and likely restricted to a subpopulation of cells (Supplementary Figure 8). However, this mutation is not directly affecting the intronic splice site and did not result in aberrantly spliced MSH6 transcripts as determined by RT-PCR. Thus, its functional role remains unclear. The functional consequences of reduced MMR protein levels for TMZ sensitivity were assessed by siRNA-mediated silencing of individual MMR genes in LNT-229 cells (Figure 5C). Down-regulation of MSH2, MSH6 or PMS2 by RNA interference led to reduced sensitivity to TMZ as assessed in clonogenic survival assays. No significant increase in resistance to TMZ was observed in LNT-229 cells after silencing of MSH3, PMS1 or MLH1.

Acquired resistance to TMZ changes chromatin organization in LNT-229 cells

It has been reported that TMZ induces heterochromatin reorganization in glioma cells (Papait *et al.* 2009). Heterochromatin is characterized by lower levels of acetylated core histones and higher levels of methylated histones, e.g., trimethylated H3K9. Immunoblots of parental and TMZ-resistant glioma cell lines revealed lower levels of acetylated H3 histones but higher levels of H3K9 trimethylated histones in resistant as opposed to parental LNT-229_R cells (Figure 6A), suggesting that a shift from a more euchromatin- to a more heterochromatin-centered status might contribute to the aquired TMZ resistance in this cell line, e.g. by altering gene expression. No such changes in histone H3 acetylation or H3K9 trimethylation were detected in the two other cell lines. Yet, altered histone acetylation does not account for the differential expression of MSH2, MSH6 and PMS2 proteins in LNT-229_R, since no change of mRNA expression levels was observed in LNT-229_R after exposure to the histone deacetylase (HDAC) inhibitor TSA (Supplementary Figure 9).

Decreased global DNA methylation levels indicated by LINE-1 methylation in LN-308_R and LN-18_R cells

Long interspersed nuclear element-1 (LINE-1) is a human retrotransposon that has been proposed to create DNA double-strand breaks with genome-destabilizing effects and may be associated with G2/M arrest in cancer cells (Gasior *et al.* 2006, Belgnaoui *et al.* 2006). In most normal cells, LINE-1 sequences are silenced by methylation, whereas in cancer cells, LINE-1 may be overexpressed (Asch *et al.* 1996, Bratthauer & Fanning 1992). Since LINE-1 accounts for approximately 18% of the human genome, its methylation status represents a surrogate indicator of global DNA methylation. Recent data suggested the LINE-1 methylation status as a better prognostic marker in glioblastoma patients than the *MGMT* promoter methylation status (Ohka *et al.* 2011). We investigated whether the methylation levels of LINE-1 differ between parental and TMZ-resistant glioma cells. We found a significant reduction of methylation in resistant LN-308_R and LN-18_R cells relative to the respective parental cells, suggesting that reduced global DNA methylation may be involved in the acquired resistance to TMZ (Figure 6B).

Alkylpurine–DNA–*N*-glycosylase (APNG) levels are not induced in resistant glioma cell lines

APNG is an enzyme that repairs the cytotoxic lesions N^3-methyladenine and N^7-methylguanine, and may contribute to TMZ resistance in glioblastoma (Agnihotri *et al.* 2012). Accordingly, we performed PCR and immunoblot analyses to measure APNG levels, but found these to be unaltered in the resistant as compared to the

corresponding parental glioma lines (Supplementary Figure 10), indicating that APNG does not play a modulating role in acquired TMZ-resistance in our cell line models.

DISCUSSION

Responsiveness to cytotoxic therapies defined by radiological responses or at least radiologically defined stable disease is commonly of short duration in malignant glioma patients. This is also true for many patients expected to derive benefit from alkylating agent chemotherapies because of *MGMT* promoter methylation (Hegi et al. 2005, Weller et al. 2010). The mechanisms leading to acquired resistance to TMZ are incompletely understood. Here, we generated TMZ–resistant glioma cell lines by repetitive exposure to increasing TMZ concentrations. Although this approach may not closely reflect the situation *in vivo*, it allows for an assessment of mechanisms triggered by repeated pulse exposure to TMZ.

First, we demonstrated that TMZ resistance can be induced in different glioma cell lines, independently of the *MGMT* promoter methylation status: the EC_{50} values increased up to 10-fold in resistant cells (Figure 1). Given the prominent role of MGMT in determining sensitivity to alkylating agent chemotherapy, it would be tempting to speculate that resistant subclones generated from *MGMT* promoter-methylated cell lines exhibit a loss of promoter methylation and *de novo* expression of MGMT. Yet, this was not the case (Figure 2A), consistent with the analysis of paired recurrent and primary tumor specimens *in vivo* which confirmed a stable *MGMT* status at recurrence in the vast majority of patients (Felsberg et al. 2011). We also investigated whether constitutively MGMT-positive LN-18 cells exhibit changes in MGMT levels when induced to become even more TMZ-resistant. Since MGMT is consumed when counteracting TMZ-induced DNA damage, the intracellular levels of MGMT are predicted to correlate with chemoresistance. In fact, we found a striking increase in MGMT mRNA and protein in TMZ-resistant LN-18 cells. Not surprisingly, LN-18_R cells exhibited cross-resistance to another alkylator, lomustine, which was not found in the MGMT-negative resistant cells that had developed MGMT-

independent pathways of resistance (Supplementary Figure 4). Immunoblot analyses revealed that MGMT levels were increased to a quantity high enough in resistant LN-18_R to still have similar levels of MGMT after exposure to 1000 μM TMZ as untreated parental cells (Figure 2B). Considering that the median serum concentration measured in patients treated with TMZ at 150 mg/m^2 in clinical settings corresponds to 50 μM (Hammond *et al.* 1999), it becomes clear how this gain in MGMT levels may confer acquired resistance. Depletion of MGMT with O^6-BG largely restored TMZ sensitivity although the resistance of LN-18_R remained slightly above that of parental cells, underlining that chemoresistance is multimodal and involves more than one pathway. One additional mechanism operating in LN-18_R cells identified here is an induction of p21, a key regulator of cell cycle progression (Fig. 4) previously linked to apoptosis resistance in glioma cells (Naumann et al. 1998, Glaser et al. 2001). Admittedly, even with O^6-BG, the concentrations required to inhibit LN-18 growth *in vitro* exceeded the TMZ concentrations achieved *in vivo*, suggesting that the study of primarily *MGMT* promoter-methylated cell lines such as LNT-229 and LN-308 acquiring resistance is the more relevant paradigm.

It has been previously reported that exposure to TMZ induces cell cycle arrest rather than cell death in glioma cells (Hirose *et al.* 2001). We noted distinct changes in the cell cycle responses to TMZ upon induction of TMZ resistance: in LNT-229_R and LN-308_R cells, the G2/M arrest induced by TMZ was abrogated and altered into continuous cellular growth. Interestingly, LN-18 cells, although relatively chemoresistant, underwent cell death rather than cell cycle arrest when treated with TMZ, albeit at concentrations exceeding the clinically relevant range. Here, resistant LN-18_R cells counteracted this effect by entering G2/M cell cycle arrest, staying in a quiescent status instead of undergoing apoptotic cell death. The major candidate mediator for this evasive mechanism was identified here as increased constitutive

expression and superinduction by TMZ of p21, a negative modulator of cell cycle progression that may lead to G1-, G2- or S-phase arrest (Ogryzko *et al.* 1997). In resistant LN-18_R cells, p21 was overexpressed up to 5-fold, likely accounting for cell cycle slowing and escape from cell death, and matching the observed slower growth of resistant LN-18_R in culture in proliferation assays (Supplementary Figure 2). An even more distinct increase in p21 mRNA levels was seen in LN-18_R when exposed to TMZ, corroborating the role of this cyclin-dependent kinase inhibitor for cell survival in resistant LN-18_R cells. These results are consistent with an increase in p21 levels as a resistance mechanism leading to a G2 arrest without induction of apoptosis in glioma cells after p53 gene transfer (Gomez-Manzano *et al.* 1997).

After having ruled out that demethylation of the *MGMT* promoter accounted for acquired resistance in the *MGMT* promoter-methylated cell lines, we investigated whether MMR protein levels varied between parental and resistant cells. To date, literature on MMR in glioma has remained inconsistent. While some findings indicate a role for cellular resistance being associated with MMR deregulation (Yip et al. 2009), others reported no significant impact of MMR on resistance (Maxwell *et al.* 2008). Here, we found that, while no significant change in MMR levels was found in LN-18_R or LN-308_R cells (Supplementary Figure 7), LNT-229_R exhibited a strong down-regulation of all 6 investigated MMR proteins in TMZ-resistant cells. Experiments with siRNA-mediated knockdown proved that MMR gene silencing mediates resistance to TMZ in LNT-229 parental cells for MSH2, MSH6 and PMS2, all of them involved in the formation and heterodimerization of the α-subunit of MutS and MutL, respectively, both more relevant for base repair than the β-subunit. This is in accordance with former investigations on colon carcinoma cells where TMZ resistance increased in MMR-deficient cells (Liu et al. 1996). Most importantly, a single protein knockdown transiently around the time of the TMZ application was

sufficient to induce resistance in formerly sensitive cells. Interestingly, these data are consistent with recent findings of reduced MMR protein levels in patient tissue from glioblastoma at recurrence, identifying the same 3 MMR proteins, MSH2, MSH6 and PMS2, as being significantly down-regulated in patients at tumor recurrence (Felsberg et al. 2011). Acquired methylation of the respective MMR gene promoters does not account for the reduced expression of MMR mRNA and protein as investigated by methylation-specific PCR in our cell lines as well as in primary and recurrent glioblastoma primary tissues (Felsberg et al. 2011). Moreover, using TSA treatment, we also detected no evidence that altered histone acetylation contributes to the observed down-regulation of MMR genes in LNT-229_R, albeit these cells displayed a globally reduced acetylation and increased methylation of histone H3. The latter finding may point to a role of epigenetic gene regulation in the acquired TMZ resistance of this cell line, however, the potentially affected genes need to be identified.

None of the above-mentioned resistance mechanisms appeared to be operating in LN-308 cells. We found a potential role for reduced methylation of LINE-1 sequences in LN-308_R and LN-18_R cells, suggesting reduced global DNA methylation that might lead to reduced DNA stability as a probable resistance mechanism. In LN-18_R, this effect could contribute to the cell cycle slowing and G2/M arrest. Collectively, our results suggest that acquired resistance to TMZ in glioma cells is not mediated through a single pathway, but that multiple mechanisms may contribute to this phenotype in different cell lines. It will be crucial for adjusted therapy to get a better understanding of all the involved pathways to allow for a personalized relapse therapy once the tumor developed resistance.

Acknowledgement: The authors thank Dr. Sankar Mitra and Dr. Kishor Bhakat for providing the MGMT reporter plasmid. This study was supported by grants from the Olga Mayenfisch Foundation to PR and from Schering Plough (MSD) to MW.

Conflict of interest: The authors declare no conflict of interest.

REFERENCES

Agnihotri, S., Gajadhar, A. S., Ternamian, C. et al. (2012) Alkylpurine-DNA-N-glycosylase confers resistance to temozolomide in xenograft models of glioblastoma multiforme and is associated with poor survival in patients. *J Clin Invest,* **122,** 253-266.

Asch, H. L., Eliacin, E., Fanning, T. G., Connolly, J. L., Bratthauer, G. and Asch, B. B. (1996) Comparative expression of the LINE-1 p40 protein in human breast carcinomas and normal breast tissues. *Oncol Res,* **8,** 239-247.

Belgnaoui, S. M., Gosden, R. G., Semmes, O. J. and Haoudi, A. (2006) Human LINE-1 retrotransposon induces DNA damage and apoptosis in cancer cells. *Cancer Cell Int,* **6,** 13.

Biswas, T., Ramana, C. V., Srinivasan, G., Boldogh, I., Hazra, T. K., Chen, Z., Tano, K., Thompson, E. B. and Mitra, S. (1999) Activation of human O6-methylguanine-DNA methyltransferase gene by glucocorticoid hormone. *Oncogene,* **18,** 525-532.

Brandes, A. A., Franceschi, E., Tosoni, A. et al. (2010) O(6)-methylguanine DNA-methyltransferase methylation status can change between first surgery for newly diagnosed glioblastoma and second surgery for recurrence: clinical implications. *Neuro Oncol,* **12,** 283-288.

Bratthauer, G. L. and Fanning, T. G. (1992) Active LINE-1 retrotransposons in human testicular cancer. *Oncogene,* **7,** 507-510.

Bronner, C. E., Baker, S. M., Morrison, P. T. et al. (1994) Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. *Nature,* **368,** 258-261.

Christmann, M., Nagel, G., Horn, S., Krahn, U., Wiewrodt, D., Sommer, C. and Kaina, B. (2010) MGMT activity, promoter methylation and immunohistochemistry of pretreatment and recurrent malignant gliomas: a comparative study on astrocytoma and glioblastoma. *Int J Cancer,* **127,** 2106-2118.

Dolan, M. E., Mitchell, R. B., Mummert, C., Moschel, R. C. and Pegg, A. E. (1991) Effect of O6-benzylguanine analogues on sensitivity of human tumor cells to the cytotoxic effects of alkylating agents. *Cancer Res,* **51,** 3367-3372.

Esteller, M., Garcia-Foncillas, J., Andion, E., Goodman, S. N., Hidalgo, O. F., Vanaclocha, V., Baylin, S. B. and Herman, J. G. (2000) Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med,* **343,** 1350-1354.

Felsberg, J., Rapp, M., Loeser, S. et al. (2009) Prognostic significance of molecular markers and extent of resection in primary glioblastoma patients. *Clin Cancer Res,* **15,** 6683-6693.

Felsberg, J., Thon, N., Eigenbrod, S. et al. (2011) Promoter methylation and expression of MGMT and the DNA mismatch repair genes MLH1, MSH2, MSH6, and PMS2 in paired primary and recurrent glioblastomas. *Int J Cancer*.

Gasior, S. L., Wakeman, T. P., Xu, B. and Deininger, P. L. (2006) The human LINE-1 retrotransposon creates DNA double-strand breaks. *J Mol Biol,* **357,** 1383-1393.

Genschel, J., Littman, S. J., Drummond, J. T. and Modrich, P. (1998) Isolation of MutSbeta from human cells and comparison of the mismatch repair specificities of MutSbeta and MutSalpha. *J Biol Chem,* **273,** 19895-19901.

Glaser, T., Wagenknecht, B. and Weller, M. (2001) Identification of p21 as a target of cycloheximide-mediated facilitation of CD95-mediated apoptosis in human malignant glioma cells. *Oncogene,* **20,** 4757-4767.

Gomez-Manzano, C., Fueyo, J., Kyritsis, A. P., McDonnell, T. J., Steck, P. A., Levin, V. A. and Yung, W. K. (1997) Characterization of p53 and p21 functional interactions in glioma cells en route to apoptosis. *J Natl Cancer Inst,* **89,** 1036-1044.

Hammond, L. A., Eckardt, J. R., Baker, S. D. et al. (1999) Phase I and pharmacokinetic study of temozolomide on a daily-for-5-days schedule in patients with advanced solid malignancies. *J Clin Oncol,* **17,** 2604-2613.

Hegi, M. E., Diserens, A. C., Gorlia, T. et al. (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med,* **352,** 997-1003.

Hermisson, M., Klumpp, A., Wick, W., Wischhusen, J., Nagel, G., Roos, W., Kaina, B. and Weller, M. (2006) O6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. *J Neurochem,* **96,** 766-776.

Hirose, Y., Berger, M. S. and Pieper, R. O. (2001) p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells. *Cancer Res,* **61,** 1957-1963.

Liu, L., Markowitz, S. and Gerson, S. L. (1996) Mismatch repair mutations override alkyltransferase in conferring resistance to temozolomide but not to 1,3-bis(2-chloroethyl)nitrosourea. *Cancer Res,* **56,** 5375-5379.

Maxwell, J. A., Johnson, S. P., McLendon, R. E. et al. (2008) Mismatch repair deficiency does not mediate clinical resistance to temozolomide in malignant glioma. *Clin Cancer Res,* **14,** 4859-4868.

Naumann, U., Durka, S. and Weller, M. (1998) Dexamethasone-mediated protection from drug cytotoxicity: association with p21WAF1/CIP1 protein accumulation? *Oncogene,* **17,** 1567-1575.

Ogryzko, V. V., Wong, P. and Howard, B. H. (1997) WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. *Mol Cell Biol,* **17,** 4877-4882.

Ohka, F., Natsume, A., Motomura, K. et al. (2011) The global DNA methylation surrogate LINE-1 methylation is correlated with MGMT promoter methylation and is a better prognostic factor for glioma. *PLoS One,* **6,** e23332.

Papadopoulos, N., Nicolaides, N. C., Wei, Y. F. et al. (1994) Mutation of a mutL homolog in hereditary colon cancer. *Science,* **263,** 1625-1629.

Papait, R., Magrassi, L., Rigamonti, D. and Cattaneo, E. (2009) Temozolomide and carmustine cause large-scale heterochromatin reorganization in glioma cells. *Biochem Biophys Res Commun,* **379,** 434-439.

Phillips, W. P., Jr., Willson, J. K., Markowitz, S. D., Zborowska, E., Zaidi, N. H., Liu, L., Gordon, N. H. and Gerson, S. L. (1997) O6-methylguanine-DNA methyltransferase (MGMT) transfectants of a 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-sensitive colon cancer cell line selectively repopulate heterogenous MGMT+/MGMT- xenografts after BCNU and O6-benzylguanine plus BCNU. *Cancer Res,* **57,** 4817-4823.

Reifenberger, J., Ring, G. U., Gies, U., Cobbers, L., Oberstrass, J., An, H. X., Niederacher, D., Wechsler, W. and Reifenberger, G. (1996) Analysis of p53 mutation and epidermal growth factor receptor amplification in recurrent gliomas with malignant progression. *J Neuropathol Exp Neurol,* **55,** 822-831.

Rellecke, P., Kuchelmeister, K., Schachenmayr, W. and Schlegel, J. (2004) Mismatch repair protein hMSH2 in primary drug resistance in in vitro human malignant gliomas. *J Neurosurg,* **101,** 653-658.

Roth, W., Grimmel, C., Rieger, L. et al. (2000) Bag-1 and Bcl-2 gene transfer in malignant glioma: modulation of cell cycle regulation and apoptosis. *Brain Pathol,* **10,** 223-234.

Srivenugopal, K. S., Yuan, X. H., Friedman, H. S. and Ali-Osman, F. (1996) Ubiquitination-dependent proteolysis of O6-methylguanine-DNA methyltransferase in human and murine tumor cells following inactivation with O6-benzylguanine or 1,3-bis(2-chloroethyl)-1-nitrosourea. *Biochemistry,* **35,** 1328-1334.

Stupp, R., Mason, W. P., van den Bent, M. J. et al. (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med,* **352,** 987-996.

Wang, G., Weiss, C., Sheng, P. and Bresnick, E. (1996) Retrovirus-mediated transfer of the human O6-methylguanine-DNA methyltransferase gene into a murine hematopoietic stem cell line and resistance to the toxic effects of certain alkylating agents. *Biochem Pharmacol,* **51,** 1221-1228.

Wedge, S. R., Porteus, J. K., May, B. L. and Newlands, E. S. (1996) Potentiation of temozolomide and BCNU cytotoxicity by O(6)-benzylguanine: a comparative study in vitro. *Br J Cancer,* **73,** 482-490.

Weller, M., Stupp, R., Reifenberger, G., Brandes, A. A., van den Bent, M. J., Wick, W. and Hegi, M. E. (2010) MGMT promoter methylation in malignant gliomas: ready for personalized medicine? *Nat Rev Neurol,* **6,** 39-51.

Wischhusen, J., Naumann, U., Ohgaki, H., Rastinejad, F. and Weller, M. (2003) CP-31398, a novel p53-stabilizing agent, induces p53-dependent and p53-independent glioma cell death. *Oncogene,* **22,** 8233-8245.

Yip, S., Miao, J., Cahill, D. P., Iafrate, A. J., Aldape, K., Nutt, C. L. and Louis, D. N. (2009) MSH6 mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance. *Clin Cancer Res,* **15,** 4622-4629.

Yung, W. K., Albright, R. E., Olson, J. et al. (2000) A phase II study of temozolomide vs. procarbazine in patients with glioblastoma multiforme at first relapse. *Br J Cancer,* **83,** 588-593.

FIGURE LEGENDS

Figure 1. Repeated exposure to TMZ induces resistance in human glioma cells. A. LNT-229, LN-308 or LN-18 glioma cells were exposed to increasing concentrations of TMZ to induce acquired resistance as described in the Materials and Methods section. EC_{50} values were calculated for parental (black) and resistant (white) cell lines. B, C. Parental (black diamonds) or resistant (open squares) cells were exposed to increasing concentrations of TMZ for 72 h in serum free medium (acute growth inhibition assay, B) or for 24 h and allowed to grow 2-3 weeks in complete medium (clonogenic cell death assay, C). Cell density was assessed by crystal violet staining. Data were assessed in triplets; representative diagram (*p<0.05, **p<0.01, ***p<0.001, t-test).

Figure 2. Acquired TMZ resistance in LN-18 cells is associated with increased MGMT expression. A. Specific primer sets were used to differentiate between methylated (M) and unmethylated (U) *MGMT* promoter sequences in parental and resistant glioma cells. U= unmethylated; M= methylated. A172 glioma cells served as a control for methylated sequences, peripheral blood mononuclear cells (PBMC) as a control for unmethylated sequences (upper panel). Whole cell lysates of parental and resistant glioma cells were assessed by immunoblot for MGMT protein levels (lower panel). GAPDH was used as a control. *MGMT* mRNA expression was examined by real-time PCR. LN-18/LN-18_R cells were transiently transfected with pGL2-Luc MGMT (575/+24ML) and pRL-CMV. Reporter activity is presented in RLU units (**p<0.01 compared to parental LN-18, t-test). B. LN-18 or LN-18_R cells were exposed to TMZ for 24 h and allowed to recover in complete medium for 72 h. MGMT levels were determined as in (A). C. LN-18 or LN-18_R cells were pretreated for 2 h

with O^6-BG (50 μM) and subsequently exposed to TMZ. MGMT levels were determined by immunoblot at 72 h. D. Glioma cells were pretreated with O^6-BG (50 μM) for 2 h, exposed to TMZ for 24 h, and assessed for clonogenic survival (parental LN-18 without O^6-BG: open diamonds; parental LN-18 + O^6-BG: black diamonds; LN-18_R without O^6-BG: open squares; LN-18_R, + O^6-BG: black squares).

Figure 3. TMZ-resistant glioma cells display different patterns of cell cycle changes in response to TMZ. A. Parental (left) and resistant (right) cells were exposed to TMZ for 24 h, cultured for an additional 48 h in serum-enriched medium, fixed, permeabilized, stained with PI and analyzed by flow cytometry. Cell distribution is shown as bar graphs (striped: sub-G1, black: G1, white: S, grey: G2/M). B. Annexin-FACS was performed in LN-18/LN-18_R cells to confirm apoptotic cell death (white: anxV/PI-negative, black: anxV-positive, grey: PI-positive, checked: anxV/PI-positive).

Figure 4. p21 is differentially expressed in LN-18-R cells. A. p21 mRNA expression was assessed by real-time PCR in TMZ-resistant cells (***$p<0.001$). B. Whole cell lysates of LN-18 and LN18_R cells were assessed for p21 protein by immunoblot. GAPDH was used as a reference. C. LN-18 or LN-18_R cells were exposed to TMZ for 24 h and cultured for an additional 24 h in serum-enriched medium prior to RNA extraction. p21 mRNA expression was subsequently assessed by real-time PCR in duplicate (*$p<0.05$, effect of TMZ, $^{+}$*$p<0.05$, effect of R phenotype).

Figure 5. Acquired TMZ resistance in LNT-229 cells is associated with decreased MMR protein levels. A. The mRNA expression of MMR family members was assessed by real-time-PCR. B. MMR protein levels were assessed by immunoblot. GAPDH was used as a reference. C. LNT-229 cells were transfected with siRNA to a MMR family member or with scrambled control siRNA. Cells were assessed for MMR protein after 72 h by immunoblot. Control-transfected (black diamonds) or MMR knockdown (open squares) cells were exposed to increasing concentrations of TMZ for 24 h, 48 h after transfection, and allowed to grow 2-3 weeks in FCS-containing medium (clonogenic assay). Cell density was assessed by crystal violet staining. Data in A and C are expressed as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001, t-test).

Figure 6. Altered histone H3 acetylation and methylation status in LNT-229_R cells. A. Global histone H3 expression (H3 total) (upper row) as well as expression of acetylated H3 (middle row) and trimethylated H3K9 (lower row) were assessed by immunoblotting in the parental and the resistant glioma cells (left panel). Corresponding quantitative analysis: right panel (black: parental cell line; white: resistant cell line). Immunoblot analysis for tubulin was used as loading control for normalization. B. Global DNA methylation levels as assessed by DNA pyrosequencing analysis of LINE-1 methylation levels (*p<0.05, t-test).