Oncolytic Herpes Simplex Virus Mutants Exhibit Enhanced Replication in Glioma Cells Evading Temozolomide Chemotherapy through Deoxyribonucleic Acid Repair

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INTRODUCTION

Engineered oncolytic viruses take advantage of cancer cell mutations and induce selective destruction.1–3 We hypothesized that chemotherapy-induced tumor-protective deoxyribonucleic acid (DNA) repair proteins promote oncolytic herpes simplex virus (HSV) replication. Specifically, in this manuscript, we demonstrate that human glioma cells respond to glioma chemotherapeutic temozolomide by expressing DNA repair genes that can be targeted to improve oncolytic HSV therapy.

Glioblastomas are aggressive neoplasms resistant to current treatments, with surgery, radiation, and chemotherapy minimally altering the median survival of the disease (12–15 months) during the past decade.4 G207, an oncolytic HSV,3 has deletions of both copies of neurovirulence gene /H9253 and an inactivating mutation of U439, encoding ICP6, the HSV ribonucleotide reductase (RR) large subunit. These mutations ensure that G207 selectively replicates in and lyses dividing cells, possibly because dividing cells express mammalian RR and growth-arrest DNA damage 34 (GADD34), genes whose products are not yet fully characterized but may regulate the cell cycle5,6 and complement G207 mutations. Mammalian RR generates deoxyribonucleotides in place of HSV RR and the GADD34 carboxyl terminus substitutes for the homologous /H9253 region.7 Intratumoral G207 inoculation has efficacy in glioma animal models and a phase I clinical trial demonstrated safety of intracranial G207 inoculation in glioma patients, with partial radiological responses.3,8,9

Temozolomide, an oral alkylating agent with slight phase II clinical trial efficacy against glioblastoma,10 spontaneously converts, at nonacidic pH, into its active metabolite, 5-(3-methyltriazlen-1-yl)imidazole-4-carboxamide (MTIC), a DNA-alkylating agent that methylates guanines at O6 and N7 positions.11 O6-methylguanine is not itself lethal to cells, but pairs with thymine, triggering mismatch DNA repair, a three-step process involving: 1) mismatched base removal by N-methylpurine-DNA glycosylase (MPG)12; 2) strand cleavage by apurinic/apyrimidic endonuclease; and 3) strand breaks recruit poly(adenosine diphosphate [ADP]-ribose) polymerase (PADPRP), a nick sensor targeting the DNA repair synthetic machinery to damaged DNA.12 However, if repair fails to keep pace with DNA damage, repetitive futile rounds of mismatch repair create DNA strand breaks, activating the serine/threonine kinase, ataxia-telangectasia mutated (ATM) and Rad3-related (ATR) during S-phase.13 If ATR activation fails to cause cell cycle arrest, subsequent S-phases convert single-strand breaks into double-strand breaks, which activate serine/threonine kinase ATM. Activated ATM promotes cell cycle arrest and apoptosis.11

Unfortunately, many gliomas lack temozolomide sensitivity, primarily because of expression of DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT). MGMT, expressed by 20% of gliomas, facilitates temozolomide resistance by removing the alkyl adduct from the O6 position of guanine before mismatch repair begins.14,15 MGMT-mediated temozolomide resistance can be partially overcome with O6-benzylguanine (O6BG), an MGMT inactivator proven safe and capable of enhancing temozolomide responsiveness of MGMT-expressing gliomas in phase I clinical trials.16

Because these new therapies generate only partial responses, glioblastoma treatment requires multimodal therapy. We hypothesized that limitations in temozolomide and G207 glioma treatment could be jointly addressed by using temozolomide-induced DNA repair genes to enhance HSV replication. Although some studies found synergy between oncolytic HSV and chemotherapy,17–21 the interaction was usually not quantified or mechanistically explained. Herein, we used Chou-Talalay multiple drug-effect analysis22 to quantify the interaction between temozolomide and oncolytic HSV. We demonstrate: 1) profound synergy between temozolomide and certain oncolytic HSVs in culture; 2) that glioma p53 expression influences MGMT expression; 3) that MGMT expression determines the HSV mutation required for synergy to
occur; 4) a mechanism of interaction: enhanced HSV replication in cells surviving temozolomide treatment by expressing DNA repair genes, which varied with tumoral MGMT expression; and 5) efficacy of combined treatment in vivo with temozolomide and G207.

**MATERIALS AND METHODS**

**Plasmids**

The MGMT complementary DNA (cDNA) was obtained from American Type Culture Collection (ATCC, Manassas, VA), and subcloned into the pCDNA3 vector (Invitrogen, Carlsbad, CA). The plasmid p53-SCX contains a cDNA encoding a dominant p53 mutation. The 700-base pair human GADD34 promoter was cloned using a 30 cycle PCR (20 seconds at 98°C, 2 minutes at 68°C per cycle) with the Takara LA Taq Polymerase (Panvera Corporation, Madison, WI), with template DNA isolated from U87 cells (Trizol, according to Invitrogen protocol); forward primer 5’-CAATTGGGAGGCAAGCCGAG-3’; and reverse primer 5’-GAATTCTAAGAGCAACGAACATAATGC-3’ (Invitrogen). The PCR product was sequenced and inserted in place of the cytomegalovirus (CMV) promoter into the plasmid pCMV-EGFP (kindly provided by T. Kuroda, Charlestown, MA), generating the plasmid pGADD34-EGFP containing the enhanced green fluorescent protein (EGFP) driven by the human GADD34 promoter.

**Cell Lines**

U87, U373, and T98 human glioblastoma and Vero (African green monkey kidney) cells were obtained from ATCC. The three plasmids, pCDNA3-MGMT, p53-SCX, and pGADD34-EGFP, were transfected into U87 cells using lipofectamine according to the manufacturer’s protocol (Invitrogen). Clones were isolated in 1 mg/ml G418 (GIBCO; Carlsbad, CA). Clones from the p53-SCX transfection were screened for mutant p53 expression using an enzyme-linked immunosorbent assay (ELISA) kit (Calbiochem, San Diego, CA), with the clone expressing the largest amount of mutant p53 designated U87/mp53. Clones from the pGADD34-EGFP transfection were screened by flow cytometry for induction of fluorescence 48 hours after temozolomide treatment, with the clone exhibiting the greatest induced fluorescence designated U87/pGADD34-EGFP. Clones from the pCDNA3-MGMT transfection were screened for temozolomide sensitivity. The clone that was most temozolomide resistant was screened for MGMT messenger ribonucleic acid (mRNA) levels by real time reverse-transcriptase polymerase chain reaction (RT-PCR), and was designated U87/MGMT. Human astrocytes were obtained from ScienCell (San Diego, CA) and cultured in human astrocyte medium (ScienCell).

**Viruses**

Wild-type HSV-1 strain F (obtained from B. Roizman, University of Chicago, Chicago, IL), strain F-derived γ34.5− ICP6−LacZ+ G207,3 strain F-derived γ34.5− R3616 (provided by B. Roizman),24 wild-type HSV-1 strain KOS (obtained from D. Knipe, Harvard Medical School, Boston, MA), and KOS-derived ICP6−LacZ+ hrR3 (obtained from S. Weller; University of Connecticut, Farmington, CT)25 were grown, purified, and titered by plaque assay on Vero cells, as described.3

**Cell Culture Cytotoxicity and Chou-Talalay Analysis**

Temozolomide (Schering-Plough; Kenilworth, NJ) was dissolved in saline, cisplatin (Sigma, St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO), and MGMT inhibitor O6BG (Sigma) was dissolved in distilled water. U87, T98, U87/MGMT, or human astrocytes were incubated overnight in 96-well plates (4000 cells/well). The next day, virus and/or chemotherapy were added to cells. Temozolomide was sometimes supplemented with 100 μmol/L O6BG. Cells were incubated for 4 days. Survival was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma) according to the manufacturer’s protocol. Dose-response curves were fit to Chou-Talalay lines,22 derived from the law of mass action, and described by the equation: \[ \log(\text{fa} / \text{fu}) = \log D - \log D_m \] where fa is the fraction affected (percent cell death), fu is the fraction unaffected (percent cell survival), D is the dose, Dm is the median-effect dose (dose causing 50% of cells to be affected, i.e., 50% survival), and m is the coefficient signifying the dose-response curve shape. Chemotherapy and virus were then added in combinations in a ratio equaling the ratio of their Dms. After fitting the combined dose-response curve to a Chou-Talalay line, Chou-Talalay combination indices (CIs) were calculated for each fa using the equation CI = [(D1/DX1) + (D2/DX2) + (D1)(D2)] / [(DX1)(DX2)], where DX1 and DX2 are the chemotherapy and virus doses required to achieve a particular fa, and D1 and D2 are the doses of the two combined required to achieve the same fa. Levels of interaction are defined as follows: CI greater than 1.3 indicates antagonism, CI between 1.1 and 1.3 indicates moderate antagonism, CI between 0.9 and 1.1 indicates additivity, CI between 0.8 and 0.9 indicates slight synergy, CI between 0.6 and 0.8 indicates moderate synergy, CI between 0.4 and 0.6 indicates synergy, and CI less than 0.4 indicates strong synergy.26
Single-Step Growth Curves

U87 cells were plated at 2.5 × 10^5 cells/well into 12-well plates containing no drug, 300 μmol/L temozolomide, or 0.1 μmol/L cisplatin for 24 hours. These concentrations are nontoxic to U87 when treatment is for 72 hours. Cells were then infected with G207 at a multiplicity of infection (MOI) of 1.5 in the presence or absence of the same temozolomide or cisplatin concentrations. Cells were scraped into the medium and subjected to three freeze-thaw cycles at various time points up to 48 hours after infection. Virus titers were determined by plaque assays on Vero cells.

Quantitative Real-Time RT-PCR

U87, T98, U87/MGMT, or human astrocyte cells were treated for 48 hours with 10 μmol/L to 3 mmol/L temozolomide or 0.01–1 μmol/L cisplatin. These doses give 30 to 90% survival after 4 days, but 100% survival at 24 to 48 hours. RNA was extracted from cells with Trizol (Invitrogen). A high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) was used to generate cDNA. Real-time RT-PCR was performed on an ABI Prism 7000 (Applied Biosystems) machine using previously described human GADD34,20 MGMT,27 ATM,28 ATR,29 MPG,30 and PADPRP12 primers (Invitrogen) combined with SYBR Green Master Mix (Applied Biosystems) or primer-probe combinations for RR sub-units M1 and M2 (Applied Biosystems) and 18S ribosomal RNA (rRNA) (Applied Biosystems) combined with TaqMan Master Mix (Applied Biosystems). Relative quantification was performed using 18S rRNA as an endogenous control. All reactions began with 10 minutes at 95°C for AmpliTaq Gold activation, followed by 40 cycles at 95°C for 15 seconds for denaturation, then 60°C for 1 minute for annealing/extension.

Small Interfering RNA

To silence gene expression, duplex RNA targeting human GADD34,20 the M2 subunit of human RR,31 and control small interfering RNA (siRNA) with medium GC content (comparable to the other siRNAs used) targeting no vertebrate sequences were synthesized with d(TT) at the 3’ terminus of each strand (Invitrogen). siRNA was transfected into U87 or T98 cells using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Levels of GADD34 and RR M2 subunit mRNA relative to mock-transfected cells were assessed at 24, 48, and 72 hours after transfection using real time RT-PCR and were reduced to 27 to 30%, 3 to 7%, and 25 to 28% of baseline values, respectively. Control siRNA maintained GADD34 and RR MR subunit mRNA levels at 95 to 100% of nontransfected cells 24 to 72 hours after transfection. Knockdown of protein levels by siRNA was confirmed by Western blot analysis. Total protein from cultured cells was extracted by RIPA buffer and 30 μg of protein was separated on a 8% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel, transferred to polyvinylidene fluoride (PVDF) membrane, and incubated with antibodies to GADD34 (Imgenex Corp., San Diego, CA) or RR M2 subunit (GenWay Biotech, San Diego, CA) at 4°C overnight. Membranes were then incubated with peroxidase-conjugated secondary antibodies for 40 minutes the next day. Protein was visualized using the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Alkaline Comet Assay

Temozolomide-induced DNA damage was quantified using the alkaline comet assay. Cells were treated with varying temozolomide concentrations for 6 hours, suspended in low-melt agarose (Trevigen, Inc., Gaithersburg, MD), and placed onto comet slides (Trevigen). After the agarose solidified, the slides underwent lysis and electrophoresis according to the manufacturers’ protocol for the alkaline comet assay. After labeling nuclei with SYBR green dye according to protocol, slides were photographed using confocal microscopy, and tail moments of 50 cells per slide were calculated using CometScore software (TriTek Corporation, Sumerduck, VA).

Flow Cytometry

U87/pGADD34-EGFP cells were treated for 48 hours with varying temozolomide concentrations. Apoptosis was screened for using the Annexin V-PE Apoptosis Detection Kit (BD Biosciences, San Jose, CA). A BD FACSCalibur Flow Cytometer (BD Biosciences) was used to sort cells on the basis of phycoerythrin (PE), EGFP, and 7-amino-actinomycin D (7-AAD) positivity, with 7-AAD positive cells (dead cells) gated out of any subsequent analysis. To assess viral replication in GADD34-expressing cells, temozolomide-treated U87/pGADD34-EGFP cells were sorted on the basis of EGFP expression using a BD FACS Vantage SE Flow Cytometer (BD Biosciences). EGFP+ and EGFP- cells were then infected with G207 (MOI, 1.5), with viral yield determined as described above.

Immunohistochemistry

U87/pGADD34-EGFP cells were treated for 48 hours with 100 μmol/L temozolomide, followed by infection with G207 (MOI, 0.1) for 10 hours. Cells were then fixed in 4% paraformaldehyde, stained with mouse anti-β-galactosidase (Promega, Madison, WI), stained with Texas Red-conjugated sheep anti-mouse antibody (Amersham), and mounted and counterstained with Vectashield mounting medium with diamidino phenyl indole (DAPI) nuclear stain (Vector Laboratories, Burlingame, CA).

In Vivo Experiments

Athymic mice (20 g) were inoculated subcutaneously with 10⁶ U87 cells. Two weeks later, mice with 12 to 36 mm³
tumors were placed into treatment groups (five mice per treatment group), each with the same mean tumor volume. Mice were treated with 100 mg/kg/d temozolomide intraperitoneally for 14 days, the maximum tolerated temozolomide dose, and/or 5 \times 10^6 pfu (plaque-forming units) G207 intratumorally in 30 μL on treatment days 2 and 5. Mock-treated mice received equivalent intraperitoneal or intratumoral volumes of saline. Tumors were measured biweekly using calipers to calculate length, width, and height, with the measurer blinded to each animal’s treatment group. Tumor volume was the product of these dimensions, and fold-growth was relative to treatment day 1. Measurement of a mouse’s tumor continued until the mouse had to be killed because of excessive tumor (2.1 cm maximal dimension).

To assess viral replication in subcutaneous tumors, 20 days after subcutaneous inoculation of 10^6 U87 cells into athymic mice, when tumors had achieved volumes of 80 to 120 mm³, mice were treated intraperitoneally with saline (n = 20) or with 100 mg/kg/d temozolomide (n = 20). On the third treatment day, 5 \times 10^6 pfu G207 was inoculated intratumorally into each tumor. Animals were killed and tumors excised at 2, 4, 6, and 8 days after G207 inoculation (five mice per group per time point), with temozolomide treatment continuing until the time of killing. Tumors were weighed, cut into small pieces, suspended in a volume of phosphate-buffered saline (PBS) that was twice the tumor volume, homogenized manually, sonicated, and centrifuged. The supernatant was isolated, freeze-thawed three times, and titered on Vero cells.

For intracranial studies, athymic mice (10 mice per treatment group) were anesthetized with intraperitoneal injection of 75 mg/kg ketamine and 15 mg/kg xylazine. After making a midline sagittal incision in mice immobilized in a stereotactic apparatus, a 1-mm burr hole was drilled 1 mm anterior to and 2.5 mm right of the bregma. Two hundred thousand U87 cells in 2 μL were then injected 4.5 mm deep, using a Hamilton syringe. On days 7, 8, and 9 after injecting tumor cells, mice were treated with 100 mg/kg intraperitoneal temozolomide or saline. On day 10, mice were treated with 7 \times 10^5 pfu G207 in 2 μL of saline using the same burr hole at which tumor cells were injected. Intracranial tumor was confirmed postmortem in all mice that died. The MGH Subcommittee on Research Animal Care approved all animal protocols.

**Statistical Analysis**

Comparisons of variables (in vitro and in vivo viral yield, mean tail moment, percent apoptosis, percent of cells that were positive for β-galactosidase, and fold-growth of subcutaneous tumors) were made using a two-tailed Student’s t test. Comparisons of Kaplan-Meier curves were made using the log-rank test.

**RESULTS**

**Treatment of p53-Intact MGMT-Negative Glioma Cells with Oncolytic HSV and Chemotherapy**

For MGMT-negative p53 wild-type cultured U87 human glioma cells, Dm of 390 μmol/L temozolomide or 0.08 MOI G207 were reduced 100-fold by combined treatment (Dm, 3.9 μmol/L temozolomide; 0.0008 MOI G207), with Chou-Talalay combination indices (CI quantifies the relationship between individual versus combined doses required for a particular fraction cell death, fa) below 0.009, well below the 0.4 cutoff for strong synergy (Fig. 9.1).

To determine whether strong synergy was unique to the temozolomide plus G207 combination, cisplatin, another glioma chemotherapy, was combined with HSV. Cisplatin, an...
Table 9.1. Interactions between chemotherapy and oncolytic HSV in various glioma cell lines

<table>
<thead>
<tr>
<th>Cell line and chemotherapy agent</th>
<th>Dm drug alone</th>
<th>Range of CI values for chemotherapy plus virus</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>G207</td>
</tr>
<tr>
<td>Temozolomide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87</td>
<td>390 μmol/L</td>
<td>0.005–0.009&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>U373</td>
<td>250 μmol/L</td>
<td>0.03–0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T98</td>
<td>1844 μmol/L</td>
<td>0.96–1.23</td>
</tr>
<tr>
<td>T98 + O6BG</td>
<td>100 μmol/L</td>
<td>0.12–0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>U87/mp53 + O6BG</td>
<td>800 μmol/L</td>
<td>0.55–0.90</td>
</tr>
<tr>
<td>U87/mp53 + O6BG</td>
<td>370 μmol/L</td>
<td>0.17–0.36&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>U87/MGMT + O6BG</td>
<td>1807 μmol/L</td>
<td>0.74–0.78</td>
</tr>
<tr>
<td>U87/MGMT + O6BG + GADD34siRNA</td>
<td>360 μmol/L</td>
<td>0.16–0.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>U87 + RRsiRNA</td>
<td>260 μmol/L</td>
<td>0.79–0.85</td>
</tr>
<tr>
<td>U87 + RRsiRNA + GADD34siRNA</td>
<td>370 μmol/L</td>
<td>0.007–0.009&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>U87/MGMT + O6BG + RRsiRNA</td>
<td>365 μmol/L</td>
<td>0.02–0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>U87/MGMT + O6BG + RRsiRNA</td>
<td>260 μmol/L</td>
<td>0.81–0.89</td>
</tr>
<tr>
<td>Human Astrocytes</td>
<td>4784 μmol/L</td>
<td>1.22–2.17</td>
</tr>
</tbody>
</table>

<sup>a</sup>Interaction was measured by CI values derived from Chou-Talalay analysis. CI > 1.3 indicates antagonism, CI = 1.1–1.3 indicates moderate antagonism, CI = 0.9–1.1 indicates additivity, CI = 0.8–0.9 indicates slight synergy, CI = 0.6–0.8 indicates moderate synergy, CI = 0.4–0.6 indicates synergy, and CI < 0.4 (these combinations are marked <sup>b</sup>) indicates strong synergy. Each combination was studied in three independent experiments, the results of which had no statistically significant difference. The results of single experiments are shown above. ND, not determined.

The role of p53 mutations in the interaction of temozolomide with recombinant HSV was explored using U373, a p53-mutated MGMT-negative human glioma cell line. In contrast, cultured human astrocytes exhibited minimal temozolomide (Dm = 4784 μmol/L) or G207 (Dm = 0.99 MOI) sensitivity, and temozolomide and G207 were antagonistic when treating astrocytes (Table 9.1).

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Intercalating agent, disrupts DNA replication and/or transcription, via a different mechanism than temozolomide. Treating U87 with G207 and cisplatin demonstrated much lesser effects (CI, 0.43–0.59; Table 9.1).

To determine whether G207 mutations contributed to the strong synergy between temozolomide and G207, we treated U87 with temozolomide combined with γ34.5-deleted HSV R3616 or ICP6-mutated hrR3, or their respective wild-type parental HSV-1 strains, F and KOS. Strong synergy occurred when treating U87 with R3616 and temozolomide (CI, 0.21–0.32; Fig. 9.1A), virtually no synergy occurred when treating U87 with hrR3 and temozolomide (CI, 0.56–1.01; Fig. 9.1A), and no synergy occurred when treating U87 with temozolomide and strain F or KOS (CI, 0.72–1.19; Fig. 9.1A). Lack of synergy with other viruses does not reflect greater replication proficiency, because Chou-Talalay analysis adjusts doses to achieve comparable effects. The Dm of G207 on U87 (MOI 0.08) exceeded that of strain F without temozolomide (MOI 0.04), but became considerably lower than that of strain F with temozolomide (when combined with temozolomide: Dm G207 = MOI 0.0008; Dm strain F = MOI 0.02), indicating that temozolomide made G207 more potent than wild-type HSV (Fig. 9.1B).
We then studied U87/mp53, a transfectant expressing mutant p53. U87/mp53 had twice the temozolomide Dm of U87 (Table 9.1). Real time RT-PCR revealed that all U87-derived p53-mutated clones, including U87/mp53, had twice as much MGMT mRNA as U87, suggesting that the U87/mp53 p53 mutation increased MGMT expression, which reduced temozolomide sensitivity. U87/mp53 generated similar interactions between temozolomide and oncolytic HSV as T98 (Table 9.1).

To isolate the role of MGMT from p53 status, we investigated U87/MGMT, a transfectant retaining the wild-type p53 of U87, but possessing 8-fold more MGMT mRNA than U87 (comparable to the 11-fold more MGMT mRNA in T98 than U87), increasing the temozolomide Dm 4.6-fold (Table 9.1). U87/MGMT generated similar interactions between temozolomide and oncolytic HSV as T98 (Table 9.1).

To determine whether synergy resulted from temozolomide enhancing G207 replication in U87, the effect of chemotherapy on infectious G207 yield was determined in single-step growth experiments. By 48 hours after infection, temozolomide-treated cells yielded greater than fivefold more G207 than untreated or cisplatin-treated cells ($P = 0.005$; Fig. 9.3A). Similarly, 2 to 8 days after G207 infection of subcutaneous U87 tumors in athymic mice, tumors from mice pretreated with temozolomide produced sixfold more G207 than those treated with saline ($P < 0.01$; Fig. 9.3B).

### Increased G207 Yield from Temozolomide-Treated Infected Cells In Vitro and In Vivo

To determine whether synergy resulted from temozolomide-enhancing G207 replication in U87, the effect of chemotherapy on infectious G207 yield was determined in single-step growth experiments. By 48 hours after infection, temozolomide-treated cells yielded greater than fivefold more G207 than untreated or cisplatin-treated cells ($P = 0.005$; Fig. 9.3A). Similarly, 2 to 8 days after G207 infection of subcutaneous U87 tumors in athymic mice, tumors from mice pretreated with temozolomide produced sixfold more G207 than those treated with saline ($P < 0.01$; Fig. 9.3B).

### Temozolomide-Induced DNA Repair Genes Vary with MGMT Expression

Next, we investigated whether U87 temozolomide treatment enhanced G207 replication because of temozolomide-induced DNA repair genes, and whether the different
interactions between temozolomide and HSV mutants observed in MGMT-expressing cells reflected induction of different DNA repair genes. Using real-time RT-PCR, we investigated genes contributing to repair of temozolomide-induced DNA damage, some of which enhance HSV replication: MPG, PADPRP, ATR, and ATM.12,13,34 We also investigated GADD34 and RR, genes complementing specific HSV mutations and possibly assisting the cellular DNA damage response. Treating U87 cells with 1 mmol/L temozolomide for 48 hours increased GADD34 expression 16-fold, exceeding the effects on other assessed transcripts (Fig. 9.4A; Table 9.2). Treating U87 cells with 1 μmol/L cisplatin did not significantly increase assessed transcript expression. No assessed transcripts increased after temozolomide treatment of human astrocytes, T98, U87/MGMT, or U87/mp53 (Fig. 9.4A; Table 9.2). Temozolomide-induced GADD34 and RR M2 subunit expression in U87 and O6BG-treated T98, along with siRNA-mediated inhibited protein expression, were confirmed by Western blots (Fig. 9.4B).

GADD34 and RR Contribute to DNA Repair and Synergy
Because they were the most temozolomide-induced transcripts assessed, we investigated whether GADD34 and RR assist repair of temozolomide-induced DNA damage. The alkaline comet assay was used to measure temozolomide-induced single-strand breaks. The mean tail moment, reflecting cumulative DNA damage, of U87 cells treated for 6 hours with temozolomide varied with temozolomide concentration, whereas, in U87/MGMT, it was minimal regardless of temozolomide dose (Fig. 9.4C). O6BG-treated U87/MGMT exhibited the same increase in tail moment with temozolomide concentration seen in U87 (Fig. 9.4C). RR siRNA, which targets RR M2 subunit mRNA, and GADD34 siRNA did not alter the tail moments at multiple temozolomide concentrations in U87 and O6BG-treated U87/MGMT, respectively. Adding GADD34 siRNA to U87 cells or RR siRNA to O6BG-treated U87/MGMT cells increased the tail moment at each temozolomide concentration (P < 0.005), suggesting that GADD34 and RR prevented DNA damage accumulation, possibly by DNA repair. In addition, GADD34 and RR siRNA inhibited the synergy of G207 and temozolomide in U87 and O6BG-treated U87/MGMT cells, respectively (Table 9.1).

GADD34 Is Expressed Mostly in Nonapoptotic Cells after Temozolomide Treatment
Speculated GADD34 roles include DNA repair or apoptosis.5 Our hypothesis that temozolomide-induced GADD34 expression enhanced G207 replication could only explain the observed synergy if GADD34 expression was not limited to cells undergoing apoptosis after temozolomide treatment, because enhanced viral replication in such cells...
TABLE 9.2. Induction of genes in U87 cells, U87/MGMT cells, and human astrocytes treated for 48 hours with temozolomide, temozolomide plus O6BG, or cisplatin

<table>
<thead>
<tr>
<th>Gene</th>
<th>U87 1 mmol/L temozolomide</th>
<th>U87/MGMT 1 mmol/L temozolomide +100 μmol/L O6BG</th>
<th>U87 1 μmol/L cisplatin</th>
<th>Human Astrocytes 3 mmol/L temozolomide</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td>−1.81 ± 0.73</td>
<td>0.37 (0.17–0.47)</td>
<td>0.77 (0.63–0.95)</td>
<td>−2.89 ± 0.91</td>
</tr>
<tr>
<td>ATR</td>
<td>0.32 ± 0.18</td>
<td>0.76 (1.10–1.41)</td>
<td>0.30 (1.13–1.40)</td>
<td>−2.0 ± 0.19</td>
</tr>
<tr>
<td>GADD34</td>
<td>4.0 ± 0.31</td>
<td>16.0* (12.9–19.8)</td>
<td>2.89 (1.10–1.41)</td>
<td>0.30 ± 0.77</td>
</tr>
<tr>
<td>MPG</td>
<td>1.41 ± 0.39</td>
<td>2.66 (2.03–3.48)</td>
<td>2.21 (1.10–1.41)</td>
<td>0.90 ± 0.22</td>
</tr>
<tr>
<td>PADPRP</td>
<td>1.1 ± 0.53</td>
<td>2.14 (1.48–3.10)</td>
<td>0.32 (0.35–0.44)</td>
<td>0.76 (0.77–0.77)</td>
</tr>
<tr>
<td>RR M1</td>
<td>−1.5 ± 0.32</td>
<td>0.35 (0.28–0.44)</td>
<td>0.19 (0.14–0.66)</td>
<td>2.60 (1.00–2.80)</td>
</tr>
<tr>
<td>RR M2</td>
<td>−1.9 ± 0.51</td>
<td>0.27 (0.19–0.38)</td>
<td>0.30 (0.19–0.38)</td>
<td>2.83 (2.30–3.48)</td>
</tr>
</tbody>
</table>

The fold-induction (2^ΔΔCT) values are given as averages from a single experiment followed by standard deviations, and the fold-induction given is derived from the mean ΔΔCT, followed by lower and upper limits, which are derived from mean ΔΔCT ± one standard deviation of ΔΔCT.

*Significant inductions (more than threefold).

Real-time RT-PCR was used to determine ΔΔCT = (Ct mRNA of interest drug treated − Ct 18S rRNA drug treated) − (Ct mRNA of interest untreated − Ct 18S rRNA untreated), which enabled determination of fold-induction (2^−ΔΔCT) in treated cells relative to untreated cells.

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Fig. 9.6B. P = 0.003 or 0.0025 for single versus combined or saline-treatment, respectively; P = 0.002 for combined versus saline treatment. Athymic mice bearing orthotopic intracranial U87 tumors treated with intraperitoneal temozolomide or intratumoral G207 achieved median survivals of 30.5 (saline) or 46 to 48 (G207/temozolomide alone) days. In contrast, temozolomide preceding G207 treatment caused 100% 90-day survival (Fig. 9.6C).

**DISCUSSION**

We hypothesized that mutations enabling cancer cells to express DNA repair genes after chemotherapy treatment could be used to improve oncolytic viral therapy. We, therefore, studied the effect of glioma MGMT expression on temozolomide-induced DNA repair genes, and whether these gene products increase replication of appropriately engineered oncolytic HSVs. For cultured MGMT-negative U87 cells, synergy of γ34.5-deleted HSV with temozolomide was very strong, whereas combining G207 with cisplatin caused only moderate synergy. In contrast, cultured MGMT-expressing temozolomide-resistant cells showed no significant synergy when combining temozolomide with any HSV, but synergy between temozolomide and RR-mutated HSV arose after adding MGMT inhibitor, O6BG. The benefits of combined treatment were confirmed in vivo when athymic mice with...
intracranial U87 tumors exhibited 100% long-term survival after treatment with G207 and temozolomide, compared with virtually none with either agent alone.

GADD34 and the RR M2 subunit were the most temozolomide-induced assessed transcripts in MGMT-negative and O6BG-treated MGMT-positive gliomas, respectively. Our findings suggest that GADD34 and RR reduce temozolomide-induced DNA damage in MGMT-negative and MGMT-positive gliomas, respectively, and enhance replication of HSV viruses, respectively. Our findings suggest that GADD34 and RR reduce temozolomide-induced DNA damage in MGMT-negative and MGMT-positive gliomas, respectively, and enhance replication of 

Cisplatin, part of procarbazine-cisplatin-vincristine (PCV) glioma chemotherapy, minimally induced the assessed transcripts, a likely explanation for the minimal synergy between cisplatin and G207. Thus, mutations enabling gliomas to express DNA repair genes after temozolomide treatment can be used to improve viral oncolysis, and a genetic alteration endowing gliomas with temozolomide resistance determines which genes temozolomide induces, which, in turn, determines the genetic profile of the oncolytic HSV whose replication temozolomide enhances.

DNA damage causes cells to initiate DNA repair and stop replicating. Although exact functions of the five known GADD proteins remain unconfirmed, they are associated with apoptosis and cell cycle arrest and are upregulated by some chemotherapies. RR synthesizes nucleotide precursors using homodimeric large (M1) and small (M2) subunits. M2 is upregulated during S-phase and by certain chemotherapies, whereas M1 expression is constant during the cell cycle, diminishing only during G0 arrest. This report furthers these demonstrations that certain chemotherapies induce GADD34 and RR by demonstrating that both proteins prevent accumulation of chemotherapy-induced DNA damage, possibly through DNA repair. In addition, our demonstration of GADD34 expression primarily in nonapoptotic cells surviving temozolomide treatment suggests greater G207 replication in cells surviving temozolomide treatment through DNA repair, underscoring the complementary tumoricidal effects of these two treatments, and unveiling the novel finding that oncolytic HSV can target tumor cells evading chemotherapy through DNA repair.

Our finding that chemotherapy-induced DNA repair genes enhanced replication of specific HSV mutants is consistent with reports of DNA repair enhancing HSV replication. In contrast, DNA repair inhibits replication of adeno-
virus, another engineered oncolytic virus studied in clinical trials.\textsuperscript{34,38}

Although previous reports found that expressing wild-type p53 in p53-mutated gliomas reduces MGMT expression,\textsuperscript{39} our finding that only certain p53 mutations increased MGMT expression suggests multifactorial MGMT regulation and that MGMT may not be upregulated in all of the 20% of glioblastomas with p53 mutations.\textsuperscript{40} We found that MGMT expression determines temozolomide-induced DNA repair genes. Although one would expect O6BG-treated MGMT-expressing cells to accumulate and repair temozolomide-induced DNA damage similarly to MGMT-negative cells, previous reports demonstrated differences in the two scenarios, including temozolomide-induced apoptotic versus autophagic cell death in MGMT-negative versus O6BG-treated MGMT-positive cells, respectively.\textsuperscript{41} The different temozolomide-induced genes in these two scenarios suggest that the scenarios differ not just in cell death mechanism, but also in DNA repair mechanisms.

The 20% of gliomas expressing MGMT exhibit a 90% failure to respond rate to temozolomide, compared with the 40% failure rate of MGMT-negative gliomas.\textsuperscript{14,15} In phase I clinical trials, the MGMT-inhibitor O6BG enhanced the response of MGMT-expressing gliomas to temozolomide.\textsuperscript{16} Temozolomide induction of GADD34 in MGMT-negative cells and RR in O6BG-treated MGMT-positive cells suggests that constructs such as G207 may be ideal to combine with temozolomide because of synergy in both scenarios through induction of different complementary mammalian genes.

Dual G207 mutations increase safety, but sometimes reduce oncolysis compared with single mutated viruses.\textsuperscript{3,42} Because temozolomide causes strong synergy with specific HSVs through tumoral GADD34 or RR induction, but upregulates neither in astrocytes, antagonizing G207 replication, temozolomide enhances the potency and therapeutic window of G207. In fact, temozolomide increased G207 potency beyond that of its wild-type parental virus.

Glioma treatment with G207 and temozolomide also enhances temozolomide potency, reducing dosage and toxicity (e.g., myelosuppression). The temozolomide dose we used, the maximum tolerated murine dose (rodents exhibit slightly more temozolomide sensitivity than humans), was minimally effective alone. G207 also has a maximal in vivo dose, not from toxicity but from the large number of cells in a postsurgical glioma cavity; inefficient delivery and distribution; and viral titer limitations reflecting viral biology and production constraints. The synergy described here increases the efficacy of these lower viral MOIs.

Two suggestions emerge from the strong synergy found between specific engineered oncolytic HSVs and temozolomide. First, the concept of generating a drug-induced viral oncolysis-enhancing response selectively in tumor cells with specific mutations warrants further investigation. Second, glioma treatment with temozolomide and G207, possibly giving temozolomide before inoculating virus during surgery to take advantage of temozolomide-induced DNA repair in residual glioma cells, warrants a clinical trial.

ACKNOWLEDGMENTS

We thank Drs. Roizman, Knipe, and Weller for providing virus. This work was supported in part by National Institutes of Health Grants NS32677 (to RLM) and P30 NS045776 (to SR) for the real-time PCR core. Robert Martuza and Samuel Rabkin are consultants to MediGene AG, which has a license from Georgetown University for G207.

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