Adenovirus-Based Strategies Overcome Temozolomide Resistance by Silencing the O^6-Methylguanine-DNA Methyltransferase Promoter

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Abstract

Currently, the most efficacious treatment for malignant gliomas is temozolomide; however, gliomas expressing the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) are resistant to this drug. Strong clinical evidence shows that gliomas with methylation and subsequent silencing of the MGMT promoter are sensitive to temozolomide. Based on the fact that adenoviral proteins directly target and inactivate key DNA repair genes, we hypothesized that the oncolytic adenovirus Δ-24-RGD could be successfully combined with temozolomide to overcome the reported MGMT-mediated resistance. Our studies showed that the combination of Δ-24-RGD and temozolomide induces a profound therapeutic synergy in glioma cells. We observed that Δ-24-RGD treatment overrides the temozolomide-mediated G_2-M arrest. Furthermore, Δ-24-RGD infection was followed by down-modulation of the RNA levels of MGMT. Chromatin immunoprecipitation assays showed that Δ-24-RGD prevented the recruitment of p300 to the MGMT promoter. Importantly, using mutant adenoviruses and wild-type and dominant-negative forms of the p300 protein, we showed that Δ-24-RGD interaction with p300 was required to induce silencing of the *MGMT* gene. Of further clinical relevance, the combination of Δ-24-RGD and temozolomide significantly improved the survival of glioma-bearing mice. Collectively, our data provide a strong mechanistic rationale for the combination of oncolytic adenoviruses and temozolomide, and should propel the clinical testing of this therapy approach in patients with malignant gliomas. [Cancer Res 2007;67(24):11499–504]

Introduction

At the moment, temozolomide is the mainstay of treatment for glioblastomas (1). However, a phase 3 evaluation of temozolomide combined with radiotherapy for newly diagnosed gliomas showed that the expression of the DNA repair enzyme O(6)-methylguanine DNA methyltransferase (MGMT; ref. 2) rendered these tumors resistant to temozolomide (3). Δ-24-RGD is an oncolytic adenovirus that harbors a 24-bp deletion in the E1A region responsible for binding Rb protein (4) and presents enhanced infectivity due to the addition of an RGD-4C motif in the fiber HI loop (5). Δ-24-RGD has proven to be highly effective against mice bearing glioma xenografts and is currently awaiting clinical trial evaluation. Because each of the new pharmacologic and oncolytic viral therapies tested in gliomas shows no toxicity but generates only partial responses, glioblastoma treatment currently requires multimodal therapy. Interestingly, adenoviral proteins directly target key DNA repair genes in order to replicate efficiently (6, 7). We hypothesized that the combination of the oncolytic adenovirus Δ-24-RGD and temozolomide would effectively overcome MGMT-mediated chemoresistance.

Materials and Methods

Animal studies. U87 MG human glioma cells (5×10^5) were engrafted into the caudate nucleus of athymic mice using a guide-screw system as previously described (8). Mice received a single intratumoral injection (day 3 postimplantation) of Δ-24-RGD (10^7 plaque-forming units/animal). Temozolomide was given at 7.5 mg/kg during 5 days.

Immunohistochemical analysis. The paraffin-embedded sections of the mice brains were immunostained for antibodies specific for adenoviral E1A (Santa Cruz Biotechnology) and hexon (Chemicon International, Inc.) proteins following conventional procedures.

Cell lines and culture conditions. The glioma cell lines U87 MG and T98G were obtained from the American Type Culture Collection. Both cell lines were maintained in DMEM/F12 (1:1, vol/vol) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C.

Adenovirus construction and infection. Construction of Δ-24-RGD and viral infection has been previously described (5, 9).

Cell viability assay. U87 MG or T98G cells were seeded at a density of 5×10^3 cells per well in 96-well plates, and the next day, cells were infected with Δ-24-RGD or UV-inactivated (UVi) at multiplicities of infection (MOI) of 0.1, 1, and 5. In addition, cells were treated with temozolomide at a concentration ranging from 0.01 to 1,000 μmol/L. Cell viability was assessed 7 days later using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich), as previously described (10). Dose-response curves were analyzed using CalcuSyn Software (Biosoft). CalcuSyn fits the dose-response curves to Chou-Talalay lines (11). IC_{50} is the median-effect dose (the dose causing 50% of cells to be affected, i.e., 50% survival). After fitting the combined dose-response curve from a single representative experiment to a Chou-Talalay line, Chou-Talalay combination indices (CI) were calculated (12). A mean CI was calculated from data points with the fraction of cells affected at > 0.5 (13).

Immunoblotting. U87 MG and T98G cells were plated and 24 h later infected with Δ-24-RGD (10 MOI for U87 MG cells and 25 MOI for T98G cells) alone or in combination with temozolomide (100 μmol/L for U87 MG cells and 300 μmol/L for T98G cells). Cells were harvested 36 h after the infection, and proteins were extracted and separated in a 10% SDS-PAGE, and the membranes were probed with antibodies for E1A (Santa Cruz Biotechnology) and fiber (NeoMarkers).

Viral replication assay. The tissue culture infection dose replication assay ($TCID_{50}$) method was used to determine the final viral titration, as previously described (5). Final titers were determined as plaque-forming units, using the validation method developed by Quantum Biotechnology.

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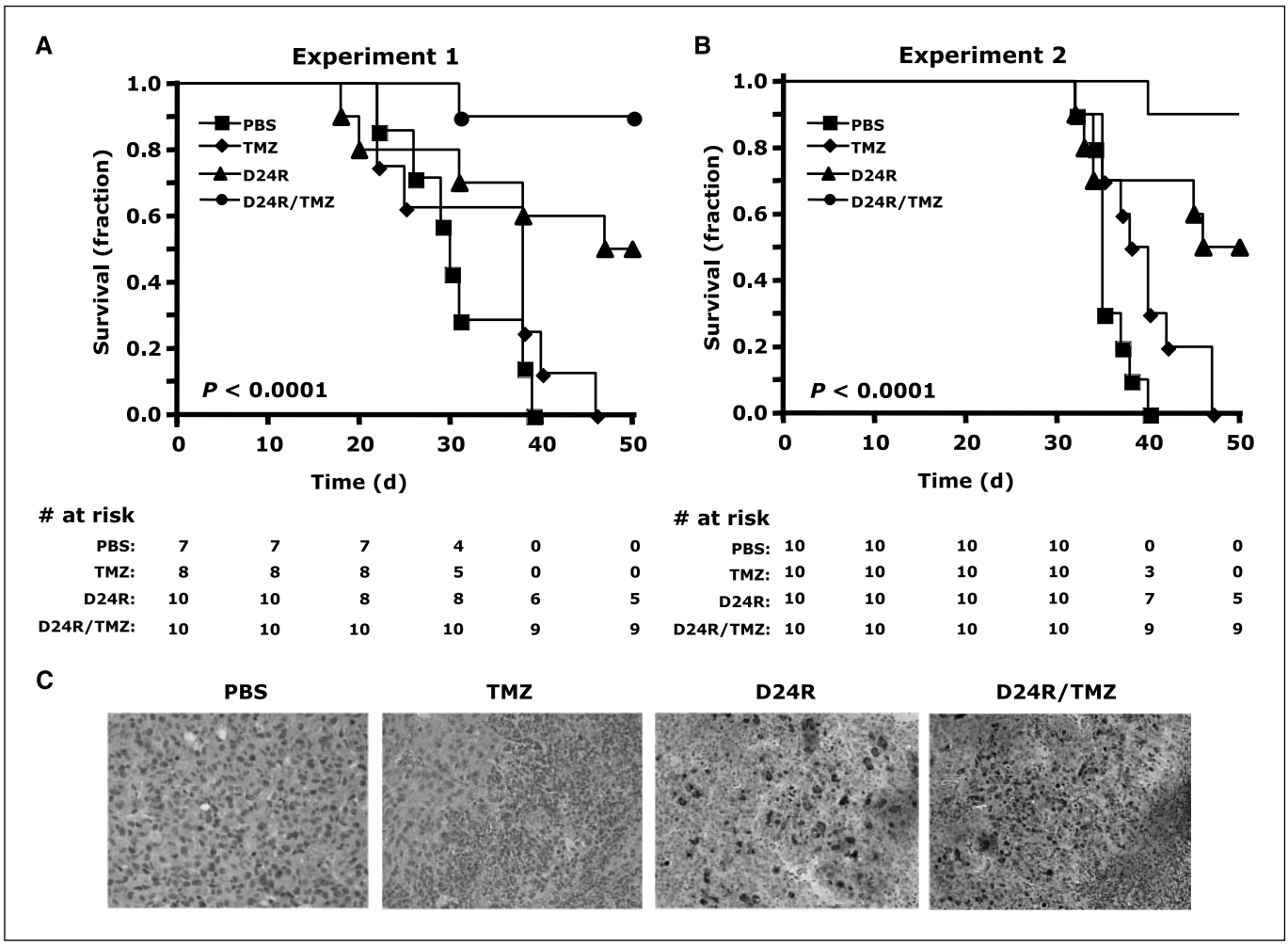

Figure 1. Temozolomide enhanced the antiglioma effect of Δ-24-RGD *in vivo*. *A* and *B,* Kaplan-Meier survival curves for overall survival in mice bearing U87 MG glioma xenografts treated with the combination of Δ-24-RGD and temozolomide (from two independent experiments). The animals were randomly distributed into four groups that received the following treatments: UViD24R and PBS (*PBS*), UViD24R and temozolomide (*TMZ*), D24R and PBS (*D24R*), and D24R and temozolomide (*D24R/TMZ*). Mice received a single intratumoral injection (day 3 postimplantation) of D24R (10^7 plaque-forming units/animal), and temozolomide was given i.p. at 7.5 mg/kg over 5 d. The *P* values in the plot were determined by the log-rank test and represent a comparison of every treatment group with that of PBS. *C,* hexon immunostaining of the brains of animals treated with Δ-24-RGD alone or in combination with temozolomide that were sacrificed 25 d after implantation. Representative photomicrographs of three animals (magnification, ×200).

Cell cycle analysis. Cell cycle phase distribution was analyzed by measuring DNA content. Cell samples were collected at different time points after infection of U87 MG or T98G cells with Δ-24-RGD (10 MOI) and/or treatment with temozolomide (100 μmol/L).

Alkaline comet assay. Cells were plated and the next day treated with Δ-24-RGD (10 and 25 MOI for U87 MG and T98G cells, respectively) and/or temozolomide (100 or 300 μmol/L for U87 MG and T98G cells, respectively). Cells were harvested 48 h after treatment, and DNA damage was quantified using the alkaline comet assay kit (Trevigen, Inc.). Data are shown as the percentage of comet tails found per treatment (n = 500 cells per treatment).

Reverse transcriptase-PCR and RT quantitative-PCR. U87 MG and T98G cells were treated with temozolomide (100 or 300 μmol/L for U87 MG and T98G cells, respectively), Δ-24-RGD (10 and 25 MOI for U87 MG and T98G cells, respectively), D39 (50 MOI), p300 (50 MOI), or AdTL2 (50 MOI) alone or in combination as indicated. Cells were collected 48 h after infection, and RNA was extracted using RNeasy (Qiagen) following the manufacturer's directions and subjected to reverse transcription-PCR (RT-PCR) and RT-quantitative (RT-QPCR). MGMT mRNA was amplified using the same primers for both types of PCR and are published elsewhere (14). In both cases, input RNA was normalized with the amplification of glyceraldehyde-3-phosphate dehydrogenase RNA. Reaction products for the RT-PCR were resolved on 2% agarose gel. QPCR analysis was performed on a Chromo 4 sequence detection system (Bio-Rad). To determine relative gene expression, the comparative threshold cycle method was used (15).

Chromatin immunoprecipitation assay. Cells were treated with the indicated single or combined treatments. The chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit (Upstate) according to the manufacturer's instructions. Cell lysates were collected 24 and 48 h after infection and immunoprecipitated with p300 antibody (Santa Cruz Biotechnology). MGMT promoter primers have been described elsewhere (16).

Statistical analysis. For the *in vitro* experiments, statistical analyses were performed using a two-tailed Student's t test. Data are expressed as mean ± SD. The *in vivo* cytopathic effect of Δ-24-RGD on human glioma xenografts was assessed by plotting survival curves according to the Kaplan-Meier method. Survival in different treatment groups was compared using the log-rank test. The *P* values for pair-wise comparisons were calculated using the Bonferroni method.

Results and Discussion

We assessed the antiglioma efficacy of Δ-24-RGD plus temozolomide *in vivo* using an orthotopic model of human glioma

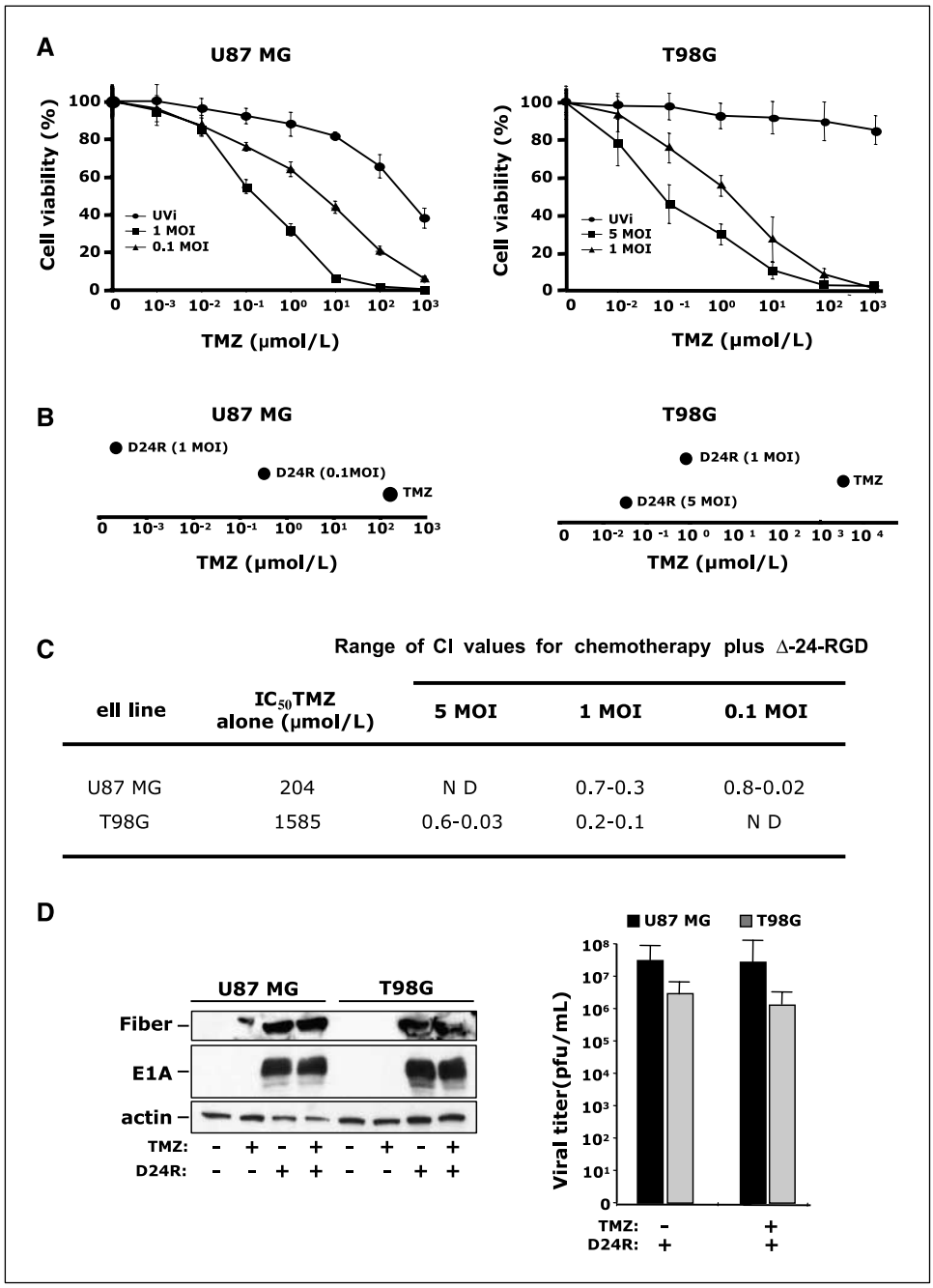

Figure 2. Characterization of the anticancer effect of the combination of temozolomide and Δ-24-RGD in gliomas. *A,* examination of the cytopathic effect of temozolomide alone or in combination with Δ-24-RGD. Cells were seeded at a density of 5×10^3 cells per well in 96-well plates. The next day, the cells were infected with D24R at a MOI of 0.1, 1, or 5 or UViD24R at a MOI of 5. Cells were treated with temozolomide at a concentration ranging from 0 to 2×10^3 μmol/L. Cell viability was assessed 7 d later. *Points,* mean of three independent experiments represented as cell viability relative to cells treated with UViD24R and temozolomide 0 μmol/L (equal to 100%); *bars,* SD. *B,* median-effect doses (IC_{50}) of temozolomide alone or in combination with Δ-24-RGD. Cells were seeded at a density of 5×10^3 cells per well in 96-well plates and the next day were infected with Δ-24-RGD at MOI of 0.1, 1, or 5 or with UVi-Δ-24-RGD at 5 MOI. Where indicated, cells were treated with temozolomide at a concentration ranging from 0 to 2×10^3 μmol/L. Cell viability was assessed 7 d later using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. IC_{50} is the median-effect dose (the dose causing 50% of cells to be affected, i.e., 50% survival). *C,* interaction between temozolomide and Δ-24-RGD in glioma cell lines. The range of CI corresponds with the different doses tested. Interaction was measured by combination index values. Combination index values >1.3 indicated antagonism, values between 1.1 and 1.3 indicated moderate antagonism, values between 0.9 and 1.1 indicated additivity, values between 0.8 and 0.9 indicated slight synergy, values between 0.6 and 0.8 indicated moderate synergy, values between 0.4 and 0.6 indicated synergy, and values <0.4 indicated strong synergy. Each combination was studied in three independent experiments, the differences of which were not statistically significant. Results of single experiments are shown. *D24R,* Δ-24-RGD; *UViD24R,* UV-inactivated Δ-24-RGD; TMZ, temozolomide; *ND,* nondetermined. *D,* analysis of the replication phenotype of Δ-24-RGD in combination with temozolomide. *Left,* expression of early and late adenoviral genes. U87 MG and T98G cells were plated and 24 h later infected with Δ-24-RGD (10 MOI for U87 MG cells and 25 MOI for T98G cells) alone or in combination with temozolomide (100 μmol/L for U87 MG cells and 300 μmol/L for T98G cells). Cells were harvested 36 h after the infection. Actin is shown as a loading control (representative immunoblot). *Right,* quantification of the replication phenotype of Δ-24-RGD in combination with temozolomide. Glioma cell lines were plated and treated with Δ-24-RGD (1 MOI for U87 MG cells and 5 MOI for T98G cells) alone or in combination with temozolomide (100 μmol/L for U87 MG cells and 300 μmol/L for T98G cells). Three days after infection, cell lysates were used to infect 293QBI cells. Viral titers were determined by the tissue culture infection dose-50 method, as described previously.

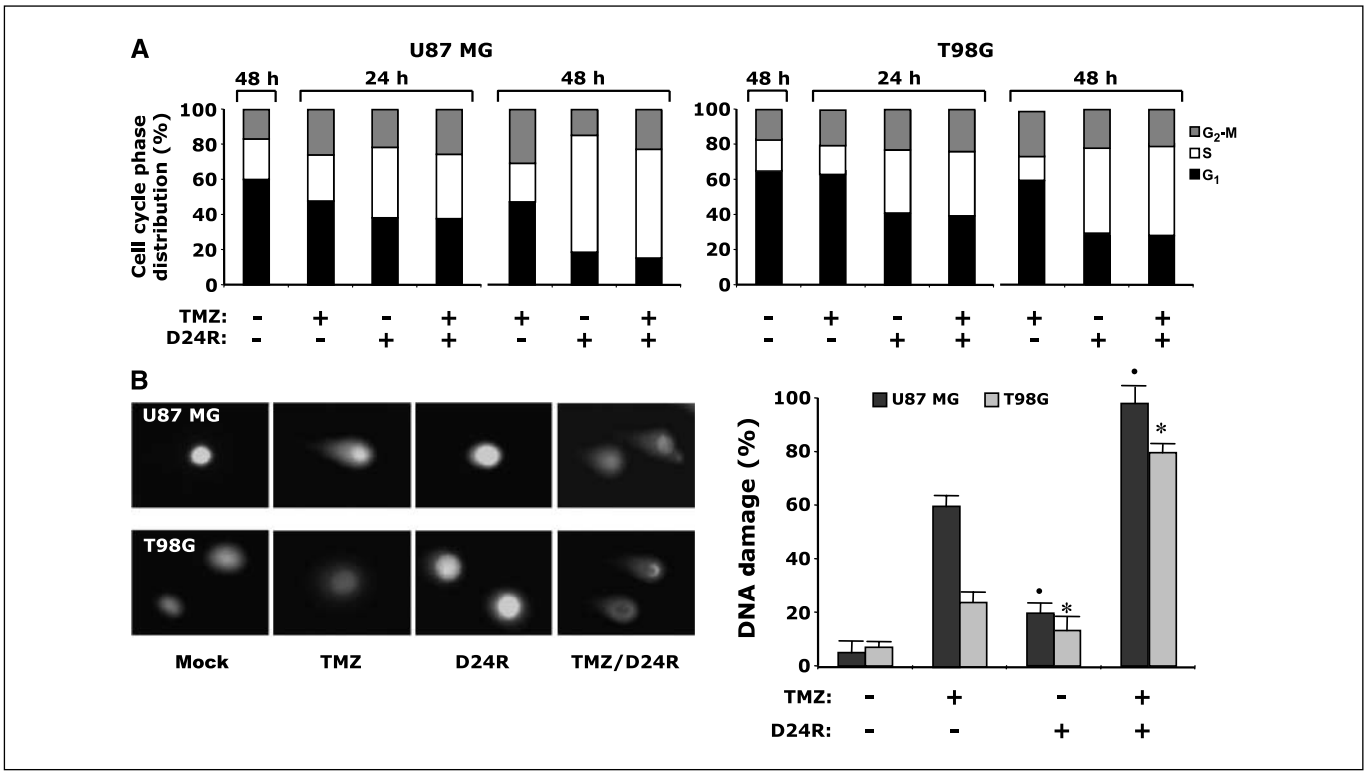

Figure 3. Role of Δ-24-RGD in overcoming temozolomide resistance in gliomas. *A,* analysis of the cell cycle profile in glioma cell lines after treatment with Δ-24-RGD and/or temozolomide. Cells were seeded and 24 h later treated with Δ-24-RGD (10 MOI for U87 MG cells and 25 MOI for T98G cells, respectively) and/or temozolomide (100 μmol/L for U87 MG cells and 300 μmol/L for T98G cells). At the indicated times, cells were harvested and cell cycle phase distribution was analyzed by measuring DNA content. *D24R,* Δ-24-RGD; *TMZ,* temozolomide. *B,* evaluation of DNA damage upon treatment with Δ-24-RGD and/or temozolomide. Cells were plated and the next day treated with Δ-24-RGD (10 and 25 MOI for U87 MG and T98G cells, respectively) and/or temozolomide (100 or 300 μmol/L for U87 MG and T98G cells, respectively). Cells were harvested 48 h after treatment, and DNA damage was quantified. *Left,* representative photomicrographs (magnification, ×200). *Right, columns,* percentage of comet tails found per treatment (n = 500 cells per treatment); *bars,* SD. All experiments were performed in triplicate and analyzed using the two-sided Student's t test (*, $P < 0.001$).

xenograft in immunocompromised mice. The median survival duration for animals treated with PBS in two independent studies was 30 and 35 days, respectively. Treatment with temozolomide alone extended the survival duration to a median 38 and 33.6 days, respectively. A single dose of Δ-24-RGD administered in combination with temozolomide significantly prolonged the survival of these mice in comparison to animals treated with Δ-24-RGD alone (P = 0.05, log-rank test for both experiments). Moreover, 50% of Δ-24-RGD–treated mice were alive 50 days after tumor implantation. Importantly, 90% of the animals treated with the Δ-24-RGD/temozolomide combination were alive at the end of both experiments (Fig. 1*A* and *B*). Next, we asked whether viral replication was impaired by the drug effect. To this end, three additional animals from each group were sacrificed 25 days after the treatment started. Animals treated with Δ-24-RGD and temozolomide showed hexon expression levels indicating that viral replication capability was not compromised in the presence of temozolomide (Fig. 1*C*).

These *in vivo* data strongly suggest that the antitumoral effect exhibited by the tandem adenovirus/chemotherapy modality was more than simple additivity. To confirm the potential synergy between Δ-24-RGD and temozolomide, we first quantified the anticancer effect of the combination of these agents in U87 MG and T98G glioma cells. We showed that the median-effect doses (IC_{50} = dose causing 50% of survival) of Δ-24-RGD were 0.57 and 4.93 MOI in U87 MG and T98G cells, respectively, and that the IC_{50} of temozolomide was 204 and 1,585 μmol/L in U87 MG and T98G cells, respectively. Importantly, we observed a decrease of between three and five orders of magnitude in the IC_{50} of the drug in the combination treatment in both cell lines (Fig. 2*A* and *B*). The analysis of the combination index values showed that the association between temozolomide and Δ-24-RGD globally resulted in a profound synergistic effect in both glioma cell lines (Fig. 2*C*).

Because temozolomide did not increase adenoviral replication (Fig. 2*D*), we next asked whether Δ-24-RGD overcomes chemoresistance to temozolomide. Because cell cycle arrest in G_2-M phase renders cells more resistant to temozolomide (17), we analyzed the cell cycle profile of U87 MG and T98G cells treated with temozolomide alone and in combination with Δ-24-RGD (Fig. 3*A*). We observed that temozolomide treatment induced G_2-M arrest but that the addition of Δ-24-RGD was sufficient to override this arrest and resulted in over-representation of the S phase populations of U87 MG and T98G cells (60.8 ± 5.7%, $P < 0.05$ and 53 ± 95%; CI, 7.2, $P < 0.005$, respectively). Because the G_2-M arrest as well as the cytotoxic mechanism of temozolomide are related to the induction of DNA damage (18), we next used the alkaline comet assay to determine whether the Δ-24-RGD infection results in the impairment of DNA repair. We showed that T98G was less susceptible than U87 MG to temozolomide-induced DNA damage (24.1 ± 3.9% and 60.2 ± 4.2%, respectively); however, the percentage of T98G and U87 MG cells with comet tails increased significantly in cells treated with Δ-24-RGD and temozolomide

(80.1 ± 3%, $P < 0.0001$ in T98G, and 98.2 ± 6%, $P < 0.0001$ in U87 MG; Fig. 3*B*). These results suggest that the adenovirus-mediated abrogation of cell cycle arrest might hinder the extra allocation of time provided by the temozolomide-induced arrest that allows the DNA repair machinery to reverse the cytotoxic effect of the drug (17), thus eventually speeding the process of cell death.

It has been proven that the expression of MGMT in cancer cells creates a resistant phenotype by blunting the therapeutic effect of temozolomide in patients with glioblastoma (3). Therefore, we evaluated the role of MGMT expression in the resistance of U87 MG and T98G cells to temozolomide. We showed that the two cell lines expressed different levels of MGMT (Fig. 4*A*), with U87 MG cells expressing low basal levels of MGMT (due to partial methylation of the promoter) and T98G cells expressing high basal levels of MGMT (promoter is wild-type). Importantly, these differences were consistent with both sensitivity to temozolomide (Fig. 2*A*) and with the extent of DNA damage induced by temozolomide (Fig. 3*B*).

Because the adenovirus E1A protein binds to and inactivates CBP/p300 and by doing so strongly inhibits MGMT promoter activity (16), we assessed MGMT expression after treatment with Δ-24-RGD alone and in combination. As expected, temozolomide treatment induced the expression of MGMT in U87 MG and T98G cells (Fig. 4*A*). However, Δ-24-RGD treatment was followed by a drastic down-modulation of MGMT transcript in both glioma cell lines treated with temozolomide (Fig. 4*A*), suggesting a critical role of p300 in these phenomena. Overexpression of p300 resulted in the up-regulation of MGMT levels in control-infected cells (>30-fold in U87 MG cells and >10-fold in T98G cells). The p300-mediated up-regulation of MGMT was drastically counteracted by Δ-24-RGD, with the levels of MGMT returning to basal levels in cells treated with both Δ-24-RGD and the adenoviral vector expressing p300. Further experiments confirmed that the expression of a dominant-negative form of p300 down-modulated the basal levels of MGMT (Fig. 4*A*). Accordingly, we showed that infection of cancer cells with a mutant E1A adenovirus (Δ-39; ref. 19) unable to bind to and inactivate p300 did not modify the level of expression of MGMT (Fig. 4*A*). Together, these data indicated that Δ-24-RGD down-modulates MGMT by means of the E1A binding to and inactivation of p300. To directly examine the effect of the physical interaction of the E1A/p300 complex with the promoter of MGMT, we performed ChIP assays. We showed that 24 h after treatment with temozolomide as single treatment or with Δ-24-RGD, p300 became a component of the protein complex recruited to the MGMT promoter in glioma cells (Fig. 4*B*). However, the recruitment of p300 to the MGMT promoter was disrupted 48 h after the infection in cells treated with Δ-24-RGD plus temozolomide (Fig. 2*B*). Our data therefore indicate that adenoviral infection is followed by E1A-mediated removal of p300 from the MGMT transcriptional machinery leading to silencing of the MGMT promoter.

Other oncolytic systems have been successfully combined with temozolomide (14, 20). Interestingly, herpes simplex virus

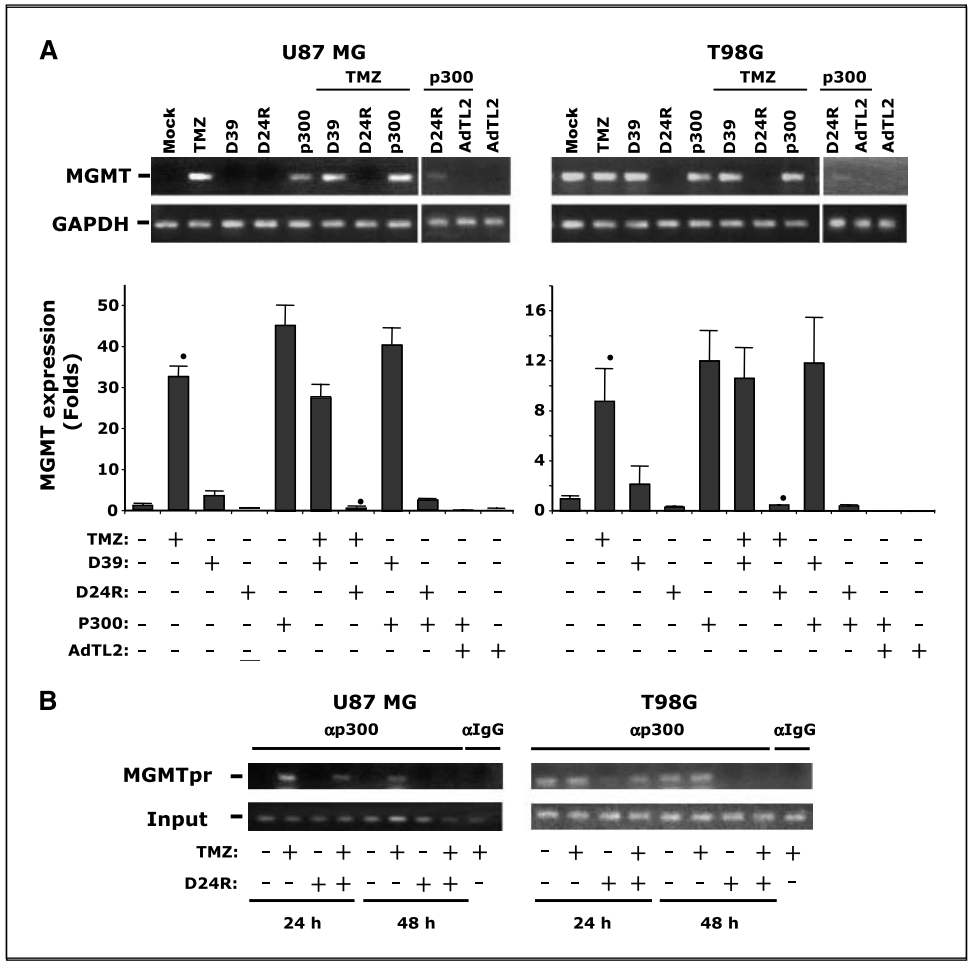

Figure 4. Role of Δ-24-RGD in overcoming temozolomide resistance in gliomas. *A,* E1A-mediated inhibition of MGMT expression. U87 MG and T98G cells were treated with temozolomide (100 or 300 μmol/L for U87 MG and T98G cells, respectively), Δ-24-RGD (10 and 25 MOI for U87 MG and T98G cells, respectively), D39 (50 MOI), p300 (50 MOI), or AdTL2 (50 MOI) alone or in combination as indicated. Cells were collected 48 h after infection, and RNA was extracted and subjected to RT-PCR (*top*) and RT-QPCR (*bottom*). In both cases, input RNA was normalized with the amplification of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) RNA. *Bars,* SD (●, $P < 0.001$ and $P = 0.02$). *B,* examination of the occupancy of the MGMT promoter by p300. Cells were treated with the indicated single or combined treatments. Cell lysates were collected 24 and 48 h after infection and immunoprecipitated with p300 antibody (Santa Cruz Biotechnology). Ten percent of the total cell lysates obtained prior to the immunoprecipitation were subjected to PCR to determine the chromatin input. *D39,* adenovirus Δ-39; *p300,* adenovirus p300; *AdTL2,* adenovirus expressing a dominant-negative form of p300.

replication benefits from the increase in the DNA damage repair machinery function triggered by the temozolomide treatment; meanwhile, adenoviruses need to shut down this machinery to achieve an effective replication.

In summary, our results indicate a synergistic therapeutic role between Δ-24-RGD and temozolomide. This work also underscores the dominant role of MGMT expression in modulating resistance to temozolomide, to date, the most active brain tumor chemotherapy. Our study suggests that therapeutic strategies targeting molecular regulation of MGMT enhances the temozolomide anticancer effect and thus link clinical observations with feasible avenues of therapy. This study also provides a preliminary rationale for a logical continuation of the imminent phase I clinical trial of Δ-24-RGD in patients with recurrent glioblastoma by proposing testing in the clinical scenario the combination of Δ-24-RGD and temozolomide in patients with malignant gliomas.

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