

BIOLOGY CONTRIBUTION

ENHANCEMENT OF GLIOMA RADIOTHERAPY AND CHEMOTHERAPY RESPONSE WITH TARGETED ANTIBODY THERAPY AGAINST DEATH RECEPTOR 5

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Purpose: TRA-8 is an agonistic mouse monoclonal antibody that binds to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor 5, which induces apoptosis in cancer cells through a caspase-8-dependent mechanism. We investigated the ability of TRA-8 to augment the radiotherapy (RT) and chemotherapy response of human glioma cells *in vitro* and *in vivo*.

Methods and Materials: The *in vitro* cytotoxicity of TRA-8 and temozolomide (Tmz) or RT was examined using adenosine triphosphate-dependent viability and clonogenic survival assays with five glioma cell lines. Death receptor 5 expression was determined by flow cytometry. *In vivo* studies included subcutaneous and intracranial xenograft models testing various combination treatments, including RT, Tmz, and TRA-8.

Results: TRA-8, combined with Tmz or RT, produced enhanced cytotoxicity against five glioma cell lines compared with the use of the individual agents alone. Death receptor 5 upregulation occurred in response to RT. Complete tumor regression in the subcutaneous experiments was the most common in animals that received combination therapy with TRA-8/Tmz/RT. TRA-8 enhanced tumor growth delay in combination with RT or Tmz. TRA-8 alone had limited activity against intracranial tumors. In contrast, the median survival of mice treated with TRA-8/Tmz/RT was significantly greater than the control or TRA-8-alone-treated mice. The median survival of the mice treated with TRA-8/Tmz/RT or chemoradiotherapy only was significantly greater than the control or TRA-8-treated mice. A trend toward improved survival was observed between TRA-8/Tmz/RT-treated and Tmz/RT-treated mice.

Conclusions: These preliminary findings support the hypothesis that TRA-8 will augment the RT and chemotherapy response in gliomas. A humanized version of TRA-8 is being evaluated in a Phase II clinical trial. © 2008 Elsevier Inc.

Tumor necrosis factor-related apoptosis-inducing ligand, TRAIL, Apoptosis, Glioblastoma multiforme, Radiotherapy, Temozolomide.

INTRODUCTION

Radiotherapy (RT) and chemotherapy modestly improve overall survival after surgical resection of malignant glioma, but newer therapies are clearly needed (1, 2). Targeting cell survival or apoptotic pathways is one strategy to improve overall survival for patients with cancer. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, Apo2L) is a member of the tumor necrosis factor superfamily that induces apoptosis after binding to membrane-bound death receptors through a caspase-8 to caspase-3-dependent mechanism (extrinsic pathway) or through caspase-8 to depolarization of mitochondria and release of cytochrome c (intrinsic pathway) (3). TRAIL plays a role in the neutraliza-

tion of activated lymphocytes, killing of virally infected cells, immune-mediated tumor cell death, and tissue turnover during embryogenesis. Five death receptors (DRs) have been described for TRAIL, including two pro-apoptotic receptors (DR4 and DR5) and three decoy receptors. The therapeutic efficacy of systemic TRAIL might be limited by the induction of apoptosis in some normal tissues, including hepatocytes. To further improve on the specificity of TRAIL-based anticancer therapies, pro-apoptotic monoclonal antibodies against TRAIL death receptors have been developed. TRA-8 is one such antibody directed against DR5. These antibodies might have clinical applications in cancer and autoimmune or inflammatory diseases (4–6). TRAIL and DR5 agonistic antibodies have activity as both pro-apoptotic

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agents and also in stimulating immune-mediated tumor surveillance (7).

Preclinical studies have demonstrated that TRA-8 does not induce apoptosis in normal human hepatocytes and does not competitively bind to decoy receptors, suggesting that it might have greater antitumor specificity than TRAIL (4). The expression of decoy receptors is tumor grade dependent in gliomas; thus, the specificity of TRA-8 might be of clinical relevance in this disease (8). Preclinical studies have demonstrated the antitumor activity of TRA-8 against a variety of tumor types as monotherapy and its enhanced tumor cell kill in combination with both RT and chemotherapy (9–14). TRAIL-mediated cell death occurs independent of oxygenation level, which could be particularly relevant in necrotic tumors such as glioblastoma multiforme (15). Temozolomide (Tmz) chemotherapy has recently been shown to improve overall survival in newly diagnosed glioblastoma multiforme when administered concurrently with and after conventional RT (16). In the present study, the preclinical therapeutic efficacy of TRA-8 alone and in combination with RT and Tmz chemotherapy was evaluated *in vitro* using five glioma cell lines and in subcutaneous and intracranial models using D54MG xenografts.

METHODS AND MATERIALS

Cells and reagents

Human glioblastoma cell lines were cultured at 37°C and 5% carbon dioxide atmosphere in Dulbecco modified Eagle medium:F12 medium with 7% fetal bovine serum (D54MG, CH235MG, U87MG), Eagle minimal essential medium with nonessential amino acids and 10% fetal bovine serum (U373MG), or Roswell Park Memorial Institute 1640 medium with 10-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM sodium pyruvate, 4.5 g/L glucose, and 10% fetal bovine serum (U251MG). Purified TRA-8 (IgG1) monoclonal antibody used for *in vitro* studies was produced and purified, as previously described (4). Daiichi Sankyo (Tokyo, Japan) provided the preparations used for the *in vivo* studies. Temozolomide (Temodar, Schering, Kenilworth, NJ) was obtained from the University of Alabama at Birmingham Hospital Pharmacy (Birmingham, AL) as 5-mg tablets, which were dissolved in dimethyl sulfoxide and centrifuged to remove insoluble material. The final dimethyl sulfoxide concentration in culture medium was <0.1%. Isotype-specific IgG1 control antibody and goat anti-mouse IgG1-phycoerythrin were from Southern Biotechnology Associates (Birmingham, AL). Collagenase type 11 and protease inhibitor cocktail were from Sigma Chemical (St. Louis, MO). The antibodies for Western blot analysis were as follows: caspase 3 and XIAP (Stressgen, Ann Arbor, MI); caspase 8 (BD Pharmingen, San Jose, CA); Bax (Southern Biotechnology Associates); Bid and Bcl-xl (Cell Signaling Technologies, Beverly, MA); p53 (Calbiochem, San Diego, CA); and actin (Sigma Chemical). Horseradish peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG were from Bio-Rad (Hercules, CA). Enhanced chemiluminescence reagents were from GE Healthcare (Piscataway, NJ).

Cell viability assays using ATPLite assay

The cytotoxicity of TRA-8 was compared with soluble TRAIL plus anti-Flag cross-linker (Alexis, San Diego, CA) using 10 glioma cell lines. The cells were plated (1,000 cells/well) in 96-well black

plates and incubated overnight before starting the treatments. For the combination treatments, the cells were pretreated with Tmz or RT or Tmz followed 1 h later by RT. TRA-8 was added 24 h later, and cell viability was assessed 5 days after starting the treatments using the ATPLite assay (Perkin Elmer Biosciences, Meriden, CT). The samples were assayed in quadruplicate and are reported as the mean \pm SD from representative experiments, which were performed at least twice.

Clonogenic survival assay

For the clonogenic survival assay, the D54MG cells were seeded in 24-well plates at 10,000 cells/well and incubated overnight. TRA-8 (39, 78, and 155 ng/mL) and Tmz (2 or 4 μ M) were added to the media for 18 h. The cells were washed, trypsinized, and replated in 6-well plates at 1,000 cells/well. The relative surviving fraction was determined 11 days later by counting colonies of >50 cells (two to four replicates per data point). The curve fitting to the experimental data was done using CurveExpert 1.3 software (available at <http://curveexpert.webhop.biz/>; Daniel G. Hyams, Hixson, TN). The inhibition concentration at 50% was calculated from regression models. The results of the clonogenic survival assays were analyzed for interaction between modalities, using the CombiTool software (Biocomputing Institut für Molekulare Biotechnologie, Jena, Germany). The analysis of synergy/antagonism of TRA-8 and Tmz was performed by comparing the theoretical and experimental effects. A theoretical dose–response surface was generated using the results obtained from the separate treatments with each individual agent. It represented the calculated additive effect of the combined doses of TRA-8 and Tmz. If the experimental data points were mapped above the theoretical surface, coincided with it, or were below it, the interaction of modalities is defined as synergistic, additive, or antagonistic, respectively (17). This method assigns a “synergy index” to each experimental data point to quantify the synergy. Zero interaction is defined when the index = 1. An index of <1 represents synergism, and of >1 represents antagonism. The results of a representative experiment are shown.

Indirect immunofluorescence and flow cytometry analysis of DR5 expression

Death receptor 5 cell surface expression was determined using flow cytometry (FACScan and CellQuest software, Becton Dickinson, San Jose, CA), as previously described (13). To examine the effect of Tmz or RT on DR5 expression, glioma cancer cell lines were treated with 10 μ M Tmz, 2 Gy RT, or 10 μ M Tmz, followed 1 h later by 2 Gy RT. The cells were harvested 24 h after starting treatment, incubated with TRA-8 and phycoerythrin-conjugated goat anti-mouse IgG1, and analyzed by flow cytometry.

Mitochondrial depolarization assay

The D54MG cells were seeded in 96-well plates at 10,000 cells/well and incubated overnight. The cells were loaded for 30 min with 10 μ g/mL JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, Eugene, OR), a cationic dye that exists as green-fluorescent monomers at low membrane potential or red-fluorescent “J-aggregates” at greater concentrations associated with greater membrane potentials. Immediately after loading, the cells were washed in phosphate-buffered saline and treated with TRA-8, Tmz, and RT. JC-1 dual fluorescence (excitation/emission = 485 nm/528 nm and excitation/emission = 485 nm/590 nm) was monitored with a Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT). A change in 590 nm to 528 nm fluorescence ratio represents the leakage of JC-1 from mitochondria.

Western blot analysis of glioma cell lines treated with TRA-8 and Tmz or RT

The cell lines were trypsinized, plated in 6-well plates (5×10^5 cells/well), and incubated overnight before starting the treatments. The cells were treated with 30 μ M Tmz or 5 Gy RT for 21 h at 37°C, then TRA-8 was added (U373MG, 5 or 25 ng/mL; CH235MG and D54MG, 25 or 125 ng/mL; and U251MG and U87MG, 125 or 1,000 ng/mL). Whole cell lysates were prepared after 3-h treatment with TRA-8 and analyzed as described previously (14).

Subcutaneous therapy experiments

Athymic nude mice were injected subcutaneously with 2×10^7 D54MG cells. When the tumors reached approximately 0.2 cm³ in volume, as determined from calculation ($0.4 \times \text{width}^2 \times \text{length}$) using two perpendicular measurements with a vernier caliper, the mice were randomized into treatment groups and the treatments started. Eight treatment groups of 7 mice each were compared: untreated control, TRA-8 alone, RT alone, Tmz alone, RT plus TRA-8, RT plus Tmz, TRA-8 plus Tmz, and TRA-8 plus Tmz plus RT (triple therapy). The mice were injected intraperitoneally with 200 μ g TRA-8 on Days 15, 18, 22, 25, 29, and 32 after tumor cell injection. The mice received 50 mg/kg Tmz by gavage on Days 16, 18, 21, 23, and 25. The tumors were irradiated with five fractions of 5 Gy (25 Gy total) of ⁶⁰Co RT given 1 h after each Tmz dose. RT was delivered to the tumor region using 8-cm lead shielding to minimize whole body exposure. In a second subcutaneous experiment, eight treatment groups with 8 animals per group were compared, but the total dose of chemoradiotherapy was lowered. Tmz was administered at 50 mg/kg on Days 14, 16, and 19, and RT was given in three fractions of 2 Gy at 1 h after Tmz administration. The primary endpoints of the subcutaneous experiments were (1) the tumor response, as determined from volume measurement described at the beginning of this paragraph, and (2) regrowth, if tumor regression occurred. All mouse studies were conducted under institutional animal care and use committee approval (APN 070106018).

Intracranial therapy experiments

The D54MG tumors were established by stereotactic intracranial injection of the mice and treated with TRA-8 alone, Tmz plus RT, or TRA-8 plus Tmz plus RT vs. the untreated controls. In each group, 8–11 animals were treated in each experiment. ⁶⁰Co RT was administered in an 8 × 32-cm collimated beam to 12 animals arranged on a 5-cm backscatter board in a head-to-head manner, with the bodies shielded from the back of the ears caudally with 8-cm-thick Cerrobend lead shielding. The primary endpoint of the intracranial experiments was survival. When the mice became moribund, as indicated by the loss of normal feeding, grooming, and avoidance behavior (neurologic signs of increasing tumor burden) or experienced a 20% decline in their initial body weight, they were killed, and that date was used to determine the survival from tumor induction. All mice underwent necropsy to ascertain that the cause of death was the intracranial tumor. In the first study, 5×10^5 D54MG cells were injected. Because of the observed weight loss during treatment, only partial therapy was administered: 200 μ g TRA-8 given by intraperitoneal injection on Days 10, 14, and 17 after tumor cell injection; three doses of 3 Gy RT given on Days 11, 14, and 16; and 50 mg/kg Tmz given 1 h before RT. The animals were then followed after treatment was discontinued. In the second intracranial experiment, the number of injected tumor cells was lowered to 2.5×10^5 . At this lower tumor burden, weight loss was not observed, and all the mice were able to complete treatment. TRA-8 was administered on Days 7, 9, 13, 16, 20, and 23 after tumor cell injection. RT was given at 3 Gy on Days 7, 9, 12, 14, and 16 at 1 h after administration of Tmz (50 mg/kg) by gavage. Survival was estimated using the Kaplan-Meier method, and comparisons between groups were performed using the log-rank test.

RESULTS

In vitro cytotoxicity of TRA-8 combined with Tmz and RT

The *in vitro* cytotoxicity of TRAIL was compared with that of TRA-8 using an adenosine triphosphate-dependent viability assay against 10 different glioma cell lines. Figure 1

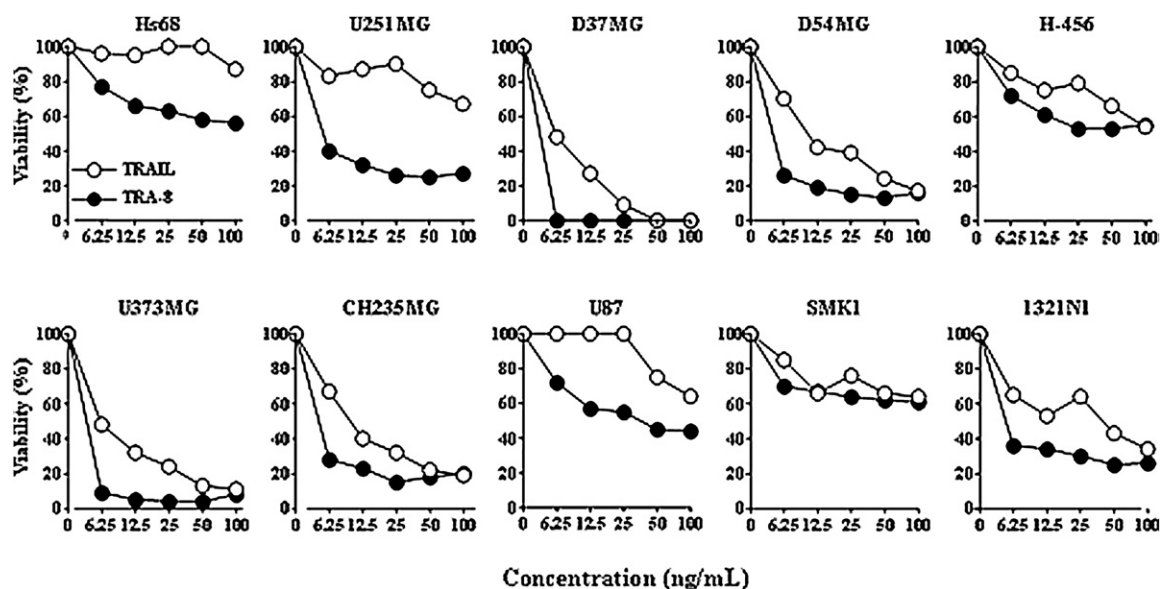


Fig. 1. Relative cytotoxicity of TRA-8 in glioma cell lines compared with that of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Cells were treated with various concentrations of recombinant TRAIL plus anti-FLAG cross-linker or TRA-8 overnight. Cell viability was determined using ATPLite assay.

shows that TRA-8 produced equal or greater cytotoxicity compared with TRAIL in all cell lines. The *in vitro* efficacy of TRA-8 combined with Tmz or RT or Tmz plus RT was examined using five human glioma cell lines of varying sensitivity to TRA-8, including sensitive (U373MG), intermediate (D54MG, CH235MG), and resistant (U251MG, U87MG) cell lines. Figure 2 shows that combination treatment with TRA-8 and Tmz produced increased killing of all five glioma cell lines after a 24-h pretreatment with Tmz followed by 96 h treatment with TRA-8 plus Tmz. Figure 3 shows that increased killing also occurred in the five glioma cell lines treated with 2, 5, or 10 Gy RT followed 24 h later by treatment with TRA-8 for 96 h. The effect of chemoradiotherapy was investigated in five glioma cell lines treated with Tmz and RT in combination with TRA-8. The results indicated enhanced cytotoxicity with the combination treatment using all three modalities compared with the individual treatments or the combinations of two modalities (Fig. 4).

Clonogenic survival assays were also used to detect enhanced *in vitro* killing of D54MG cells using TRA-8 in combination with Tmz (Fig. 5). The TRA-8-alone dose-response curve (Fig. 5) was fitted to a linear model [$y=a+bx$] (SE = 0.034, R = 0.994). The Tmz-alone curve was fitted to a sinusoidal model [$y=a+b*\cos(cx+d)$] (SE = 0, R = 1), and the curve of the combination of TRA-8 and 2- μ M Tmz was fitted to an exponential association model [$y = a(b-\exp(-cx))$] (SE = 0.021, R = 0.999). An analysis of the dose-response curves from Fig. 5 revealed that adding 2 μ M of Tmz to TRA-8 resulted in lowering the TRA-8 inhibition concentration at 50% values from 126.8 ng/mL to 9.8

ng/mL. In contrast, the inhibition concentration at 50% value for Tmz alone was 3.2 μ M. An analysis of the synergy or antagonism of TRA-8 and Tmz combination treatment (Fig. 5) was performed, and an interaction index was assigned to each experimental data point. For TRA-8 (39, 78, and 156 ng/mL) combined with 2- μ M Tmz, an interaction index of 0.570, 0.608, and 0.694 was calculated, respectively, indicating that the strongest synergistic interaction occurred at the lowest TRA-8 dose.

Mechanisms of chemotherapy and RT enhancement of TRA-8 induced cytotoxicity

The induction of apoptosis is associated with a disruption in mitochondrial membrane integrity and a subsequent reduction in transmembrane potential. Mitochondrial membrane depolarization was investigated in response to treatment with TRA-8 alone and in combination with Tmz or RT using the fluorescent dye JC-1, which differentially stains aggregates of the dye red under conditions of high mitochondrial potential and stains monomeric JC-1 molecules green under conditions of low membrane potential. The ratio of JC-1 aggregates to monomers in the D54MG cells was reduced by 21 h of treatment with TRA-8, indicating a loss in mitochondrial membrane potential (Fig. 6). Neither 100 μ M of Tmz nor 3 Gy of RT contributed to the reduction of mitochondrial membrane potential relative to TRA-8 alone.

Chemotherapy- or RT-induced enhancement of DR-mediated apoptosis could occur through several mechanisms, including upregulation of DR5 expression. Flow cytometry was used to examine DR5 expression in glioma cell lines

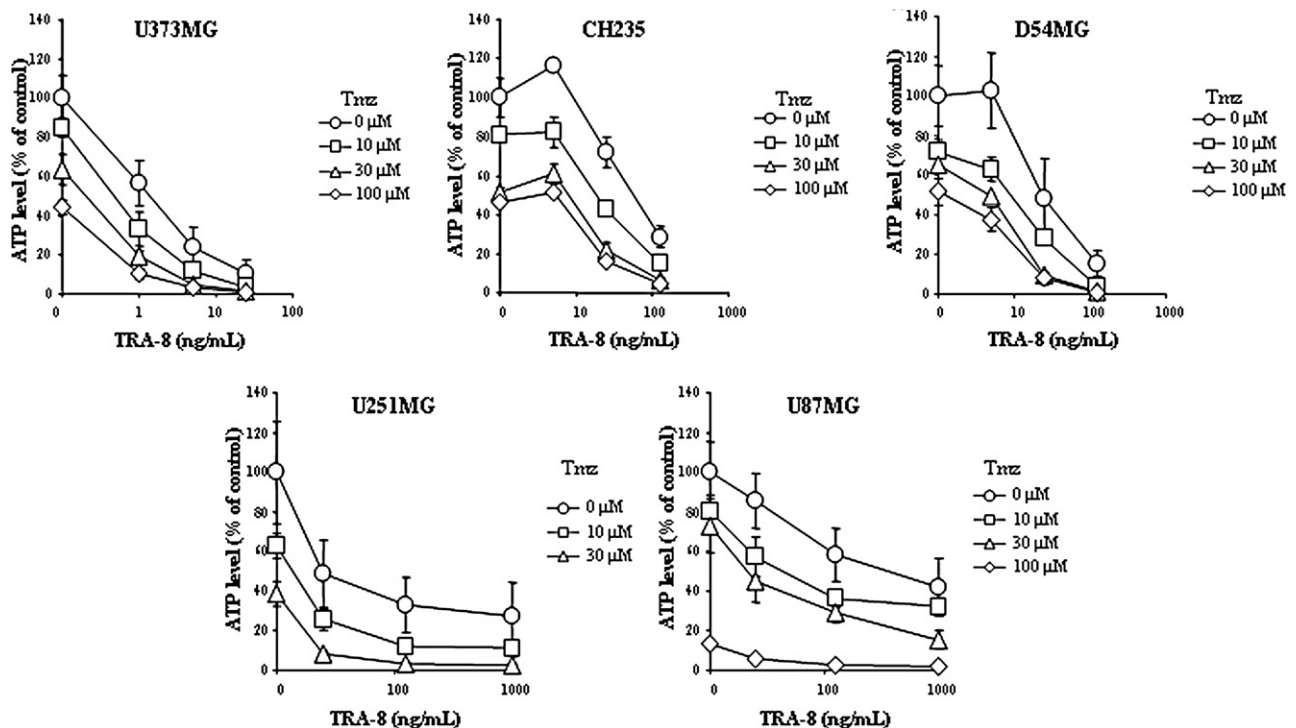


Fig. 2. Cytotoxicity of TRA-8 combined with temozolomide treatment of glioma cell lines. Cells were treated with 0–100 Tmz for 24 h followed by 96 h treatment with TRA-8 alone, Tmz alone, or TRA-8 plus Tmz. Adenosine triphosphate (ATP) levels were determined 5 d after start of treatment.

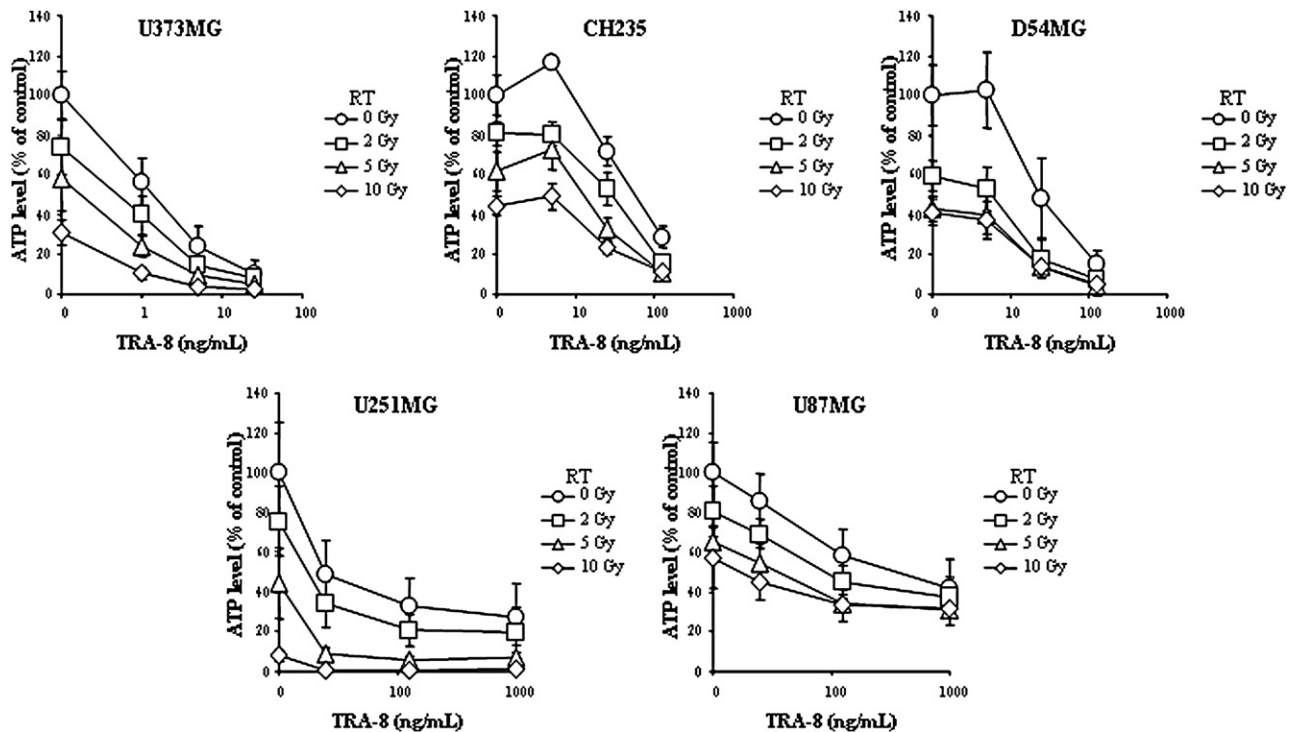


Fig. 3. Cytotoxicity of TRA-8 combined with radiotherapy (RT) of glioma cell lines. Cells were treated with 0–10 Gy RT, and TRA-8 was added 24 h later. Adenosine triphosphate (ATP) levels were determined after 5 d of treatment.

after treatment with Tmz or RT or Tmz plus RT. DR5 expression was not increased in D54MG, U251MG, or CH235MG cells after 24 h of treatment with 10 or 30 μ M Tmz (Fig. 7; and data not shown). Treatment with 2 or 5 Gy of RT modestly increased DR5 expression in D54MG cells, but DR5 levels were not increased by the combination of Tmz and RT compared with RT alone. Little or no change in DR5 expression was detected in U251MG, CH235MG or U87MG cells at 24 h after treatment with RT or Tmz followed by RT (data not shown).

Alterations in other apoptotic regulatory molecules were investigated in five glioma cell lines to further explore the mechanisms of response to TRA-8 combined with Tmz or RT. Caspase 8 cleavage has been shown to be a critical event in the induction of DR5-mediated apoptosis, resulting in the activation of downstream molecules, including caspase 3. Treatment with TRA-8 alone for 3 h resulted in cleavage of caspase 8 and caspase 3 in all five cell lines, although the required dose of TRA-8 and the levels of caspase cleavage products varied with the sensitivity of the cell lines to TRA-8 (Fig. 8). Treatment with 5 Gy of RT increased caspase 8 cleavage, as shown by a reduction in procaspase 8 levels in CH235MG and U87MG glioma cells, compared with treatment with TRA-8 alone. Treatment with TRA-8 alone also reduced Bid levels, presumably because of caspase 8-dependent cleavage. In contrast, treatment with 5 Gy of RT alone upregulated Bid in three glioma cell lines. A modest reduction in anti-apoptotic Bcl-x1 protein levels was detected in U251MG and U87MG cells after treatment with TRA-8 alone and when combined with Tmz or RT. In contrast,

Bcl-x1 levels were very high in the CH235MG and D54MG cells (Fig. 8). Slightly increased Bax levels were detected in U373MG, CH235MG, and D54MG cells treated with TRA-8 and RT. However, the p53 levels did not change appreciably after treatment with TRA-8, Tmz, or RT, suggesting that p53 upregulation does not contribute significantly to the enhanced killing by TRA-8.

Subcutaneous *in vivo* therapy experiments

The antitumor efficacy of various combinations of TRA-8, Tmz, and RT was evaluated compared with the untreated controls in an eight-arm experiment using D54MG subcutaneous xenografts. The mean tumor size in each treatment group at the initiation of treatment was similar (8 mm in diameter). The only tumors to completely regress had received TRA-8, including all seven tumors treated with the triple therapy of TRA-8, Tmz, and RT (Fig. 9a). The mean time to complete regression of the tumors was 71 days (range, 33–99), even though all therapy was completed by Day 33. TRA-8 alone had limited activity as a single agent in this model. The results were similar in a second subcutaneous experiment using a lower chemoradiation dose (Fig. 9b). Combining both subcutaneous experiments, complete tumor regression was most common in mice that received the triple therapy combination of TRA-8/Tmz/RT (12 of 15 animals) compared with those that did not (4 of 112 animals; $p < 0.001$). Three of the four mice with complete regression that did not receive triple therapy received TRA-8/Tmz.

The addition of TRA-8 also enhanced tumor growth delay in combination with either RT or Tmz alone. Tumor growth

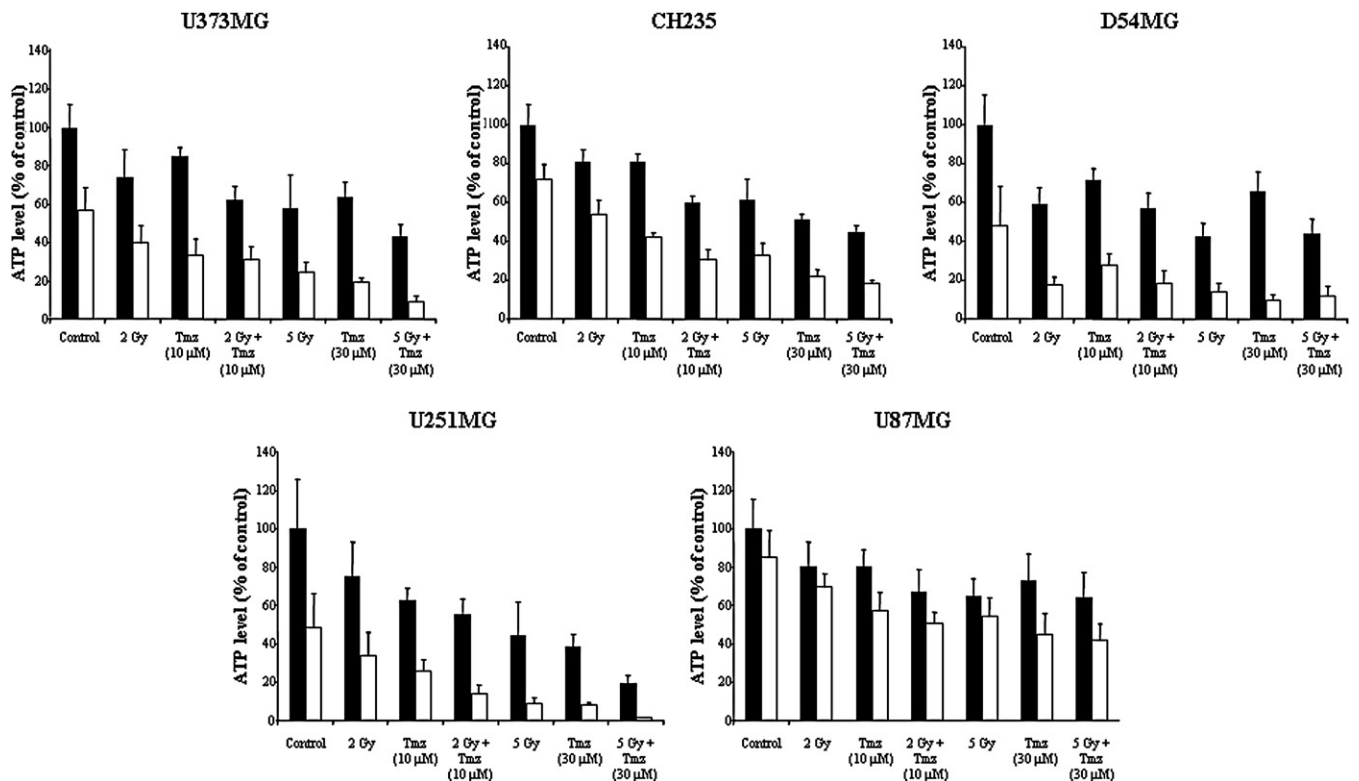


Fig. 4. Cytotoxicity of TRA-8 combined with temozolomide (Tmz) and radiotherapy (RT) of glioma cell lines. Cells were treated with 10 μ M Tmz followed 1 h later by 2 Gy of RT or treated with 30 μ M Tmz followed by 5 Gy; TRA-8 was added 24 h after RT. Adenosine triphosphate (ATP) levels were determined after 5 d of treatment. Closed bars represent cells incubated without TRA-8. Open bars represent cells treated with TRA-8 at 1 ng/mL (U373MG), 25 ng/mL (CH235MG, D54MG, U251MG), or 1,000 ng/mL (U87MG).

curves are shown in Fig. 9 for the two subcutaneous experiments. The addition of TRA-8 to either RT or chemotherapy delayed tumor growth compared with RT or chemotherapy alone. On long-term follow-up in the first subcutaneous experiment (Fig. 9a), selected tumors that did not regrow after

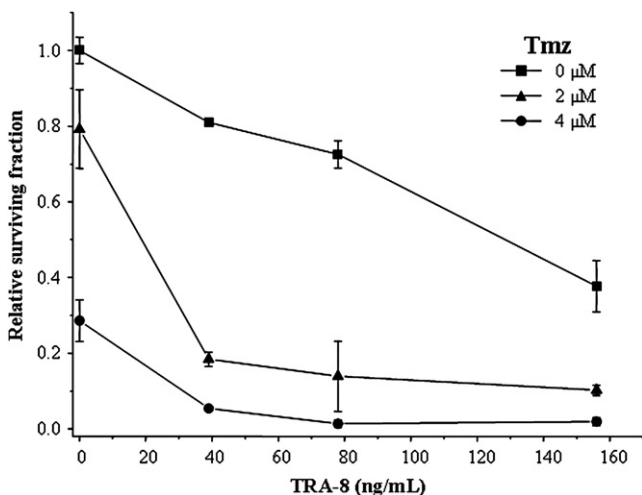


Fig. 5. Effect of TRA-8 and temozolomide (Tmz) on clonogenic survival of D54MG cells. Cells were treated with TRA-8 and Tmz for 18 h then trypsinized and replated. Viable colonies (>50 cells/colony) were counted after 11 d of growth. Surviving fraction calculated by normalizing number of colonies present after Tmz treatment to untreated control.

treatment with chemoradiotherapy underwent biopsy and were found to be sterile despite what appeared to be residual gross tumor. Thus, this first subcutaneous experiment did not confirm that triple therapy, including TRA-8, was better than Tmz plus RT without TRA-8. TRA-8 with Tmz or RT did enhance the inhibition of tumor growth compared with Tmz and RT individually. However, in the second

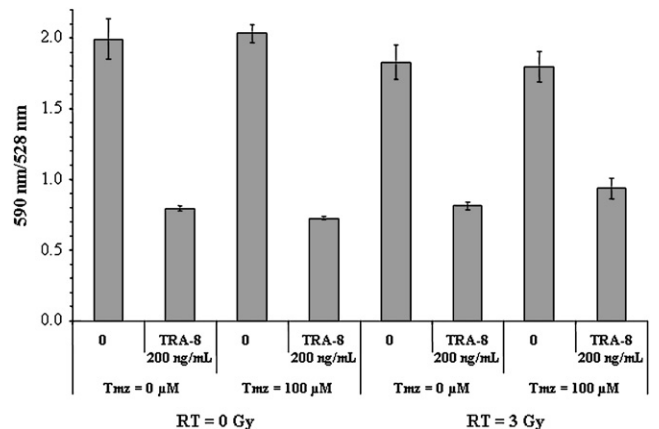


Fig. 6. TRA-8 induced depolarization of mitochondria in D54MG cells. Cells were treated with 200 ng/mL TRA-8, 100 μ M temozolomide (Tmz), 3 Gy radiotherapy (RT), or combinations of TRA-8, Tmz, and RT. Ratio of JC-1 aggregates (red fluorescence) to monomers (green fluorescence) was determined 21 h after start of treatment.

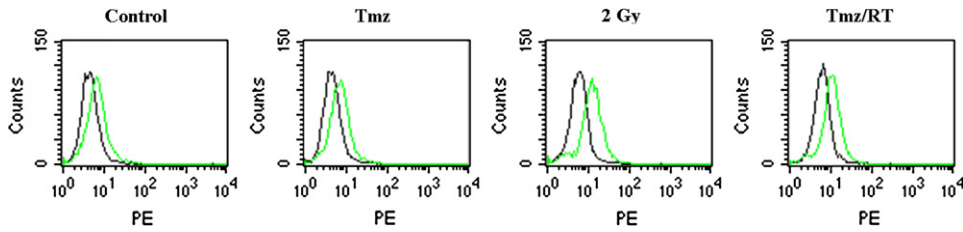


Fig. 7. Death receptor (DR)5 cell surface expression in D54MG glioma cells treated with temozolomide (Tmz) and/or radiotherapy (RT). Cells were treated with 10 μ M Tmz, 2 Gy RT, or Tmz followed 2 h later by 2 Gy RT. Cells were harvested 24 h after starting treatment, stained with TRA-8 (green lines) or mouse IgG1 isotype control antibody (black lines) followed by goat anti-mouse IgG1-phycoerythrin, and analyzed by flow cytometry. PE = phycoerythrin.

subcutaneous experiment using a lower chemoradiation dose, a difference was observed in the tumor regrowth between chemoradiotherapy alone and chemoradiotherapy with the antibody (Fig. 9b). The sample size of this experiment was insufficient for statistical analysis.

Intracranial in vivo therapy experiments

The interaction of TRA-8, Tmz, and RT was evaluated in a D54MG intracranial xenograft model with overall survival as the primary endpoint. In the intracranial glioma experiments, TRA-8 alone had marginal activity, with significantly

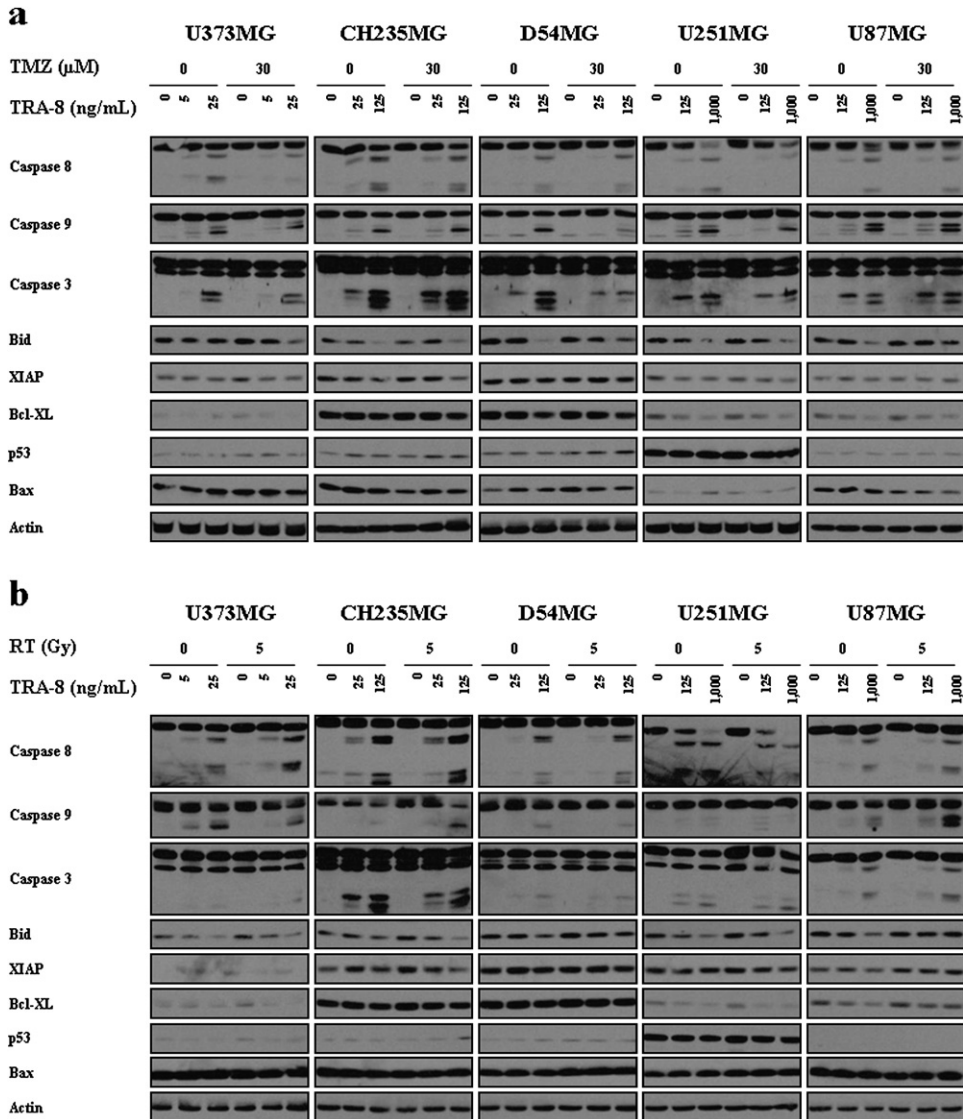


Fig. 8. Alterations in levels of pro- and anti-apoptotic proteins in glioma cell lines treated with TRA-8 combined with temozolomide (Tmz) or radiotherapy (RT). Cells were treated with (a) 0 or 30 μ M Tmz and (b) 0 or 5 Gy RT. TRA-8 was added 21 h after Tmz or RT. Whole cell lysates were prepared after 3 h treatment with TRA-8 and analyzed by Western blot.

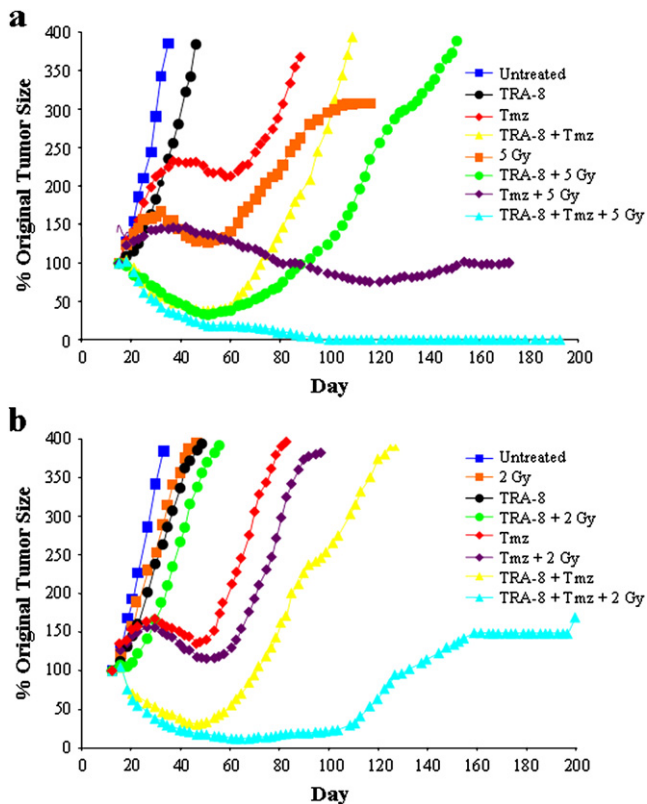


Fig. 9. TRA-8 induced D54MG tumor regression and regrowth in subcutaneous tumor models. (a) Athymic nude mice were injected subcutaneously with 2×10^7 D54MG cells. Eight treatment groups were compared: untreated control, TRA-8 alone, radiotherapy (RT) alone, temozolomide (Tmz) alone, RT plus TRA-8, RT plus Tmz, TRA-8 plus Tmz, and TRA-8 plus Tmz plus RT (triple therapy). Primary endpoints were tumor response and regrowth. (b) Repeat of experiment performed using lower chemoradiotherapy dose.

different median survival times in the studies in which different numbers of tumor cells were injected. In both studies, the median survival of the triple-treated or chemoradiotherapy-only treated mice were significantly greater than that of the control or TRA-8-alone mice (Fig. 10). Although the median survival of the triple-treated mice (107 and 219 days) was greater in both studies than that of mice receiving chemoradiotherapy without TRA-8 (75 and 166 days), these differences were not statistically significant on log-rank analysis of the Kaplan-Meier survival estimations ($p = 0.10$ and $p = 0.17$), respectively.

DISCUSSION

Targeted pro-apoptotic therapies hold great promise in the treatment of cancer. As early as 1999, intracranial experiments suggested that locally administered TRAIL might be efficacious against malignant gliomas (18). Systemic TRAIL administration might not be feasible because of the potential toxicity; however, this has been debated because different preparations of recombinant TRAIL have differential hepatocyte toxicity (4, 19–21). This potential limitation led to the development of pro-apoptotic monoclonal antibodies against

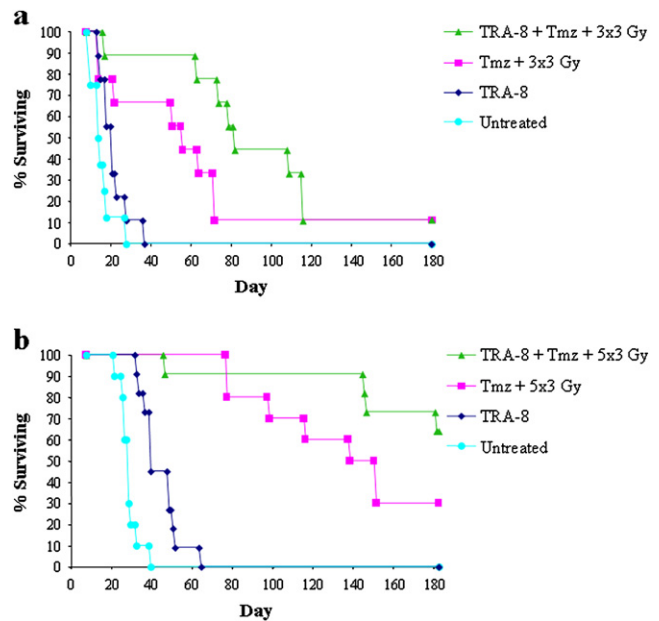


Fig. 10. Overall survival in D54MG intracranial model. (a) Tumors were established by stereotactic intracranial injection of 5×10^5 D54MG cells in mice and treated with TRA-8 alone, temozolomide (Tmz) plus RT, or TRA-8 plus Tmz plus RT (3 Gy in three fractions) vs. untreated control. Primary endpoint was survival. (b) In second intracranial experiment, burden of injected tumors cells was lowered to 2.5×10^5 .

individual DRs (22–29), including TRA-8, which binds to DR5 (4). TRA-8 has potential application in several tumor types in which enhancement of chemotherapy and RT has been observed preclinically (9–14). A humanized version of TRA-8 was evaluated in a Phase I study and is under evaluation in a Phase II clinical trial.

The *in vitro* studies presented in the present report demonstrated that combination treatment with Tmz or RT increased TRA-8 cytotoxicity against a panel of established glioma cell lines. The results of the *in vivo* experiments support the hypothesis that TRA-8 would enhance both RT and Tmz chemotherapy in a human glioma xenograft model, although a larger sample size is required to demonstrate the statistical significance of these observations. RT increased DR5 expression on D54MG cells as detected by TRA-8 binding and flow cytometry, but no change in DR5 expression was detected in three other glioma cell lines treated with Tmz or RT, indicating that other mechanisms contribute to the enhanced TRA-8 activity. TRA-8-induced cleavage of caspase 8 was detected in all five glioma cell lines, which correlated with reduced levels of Bid, presumably because of cleavage by caspase 8, an event linked to activation of the intrinsic mitochondrial apoptotic pathway (30). RT induced a modest increase in Bid levels in three glioma cell lines, which might have contributed to activation of the intrinsic pathway. RT also increased Bax levels in U373MG, CH235MG, and D54MG cells, which might have enhanced TRA-8 killing, because Bax is important for TRAIL-mediated apoptosis (31). However, no direct correlation between the TRA-8 response and the levels of p53 or Bax was observed in the five glioma cell

lines. Likewise, a modest reduction in the levels of the anti-apoptotic Bcl-x1 protein was detected in glioma cells treated with TRA-8 alone and combined with Tmz or RT, but no direct correlation between the basal levels of Bcl-x1 and TRA-8 response was observed, because the CH235MG and D54MG cells expressed high levels of Bcl-x1 but were sensitive to TRA-8. These results indicate that more detailed mechanistic studies are required to identify the factors responsible for the sensitivity or resistance to TRA-8-mediated apoptosis.

Previous investigations of TRAIL resistance reported no correlation with DR4 or DR5 expression between sensitive or resistant glioma cell lines (32). TRAIL sensitivity has been correlated with levels or localization of various proteins, including Akt, FLIP, and c-myc (33, 34). Several investigators have demonstrated increased activity of TRAIL-based therapies combined with alkylating agents clinically used to treat malignant gliomas. Röhn *et al.* (32) found that several chemotherapeutic drugs, including lomustine and Tmz, combined with TRAIL in U87MG glioma cells synergistically enhanced cytochrome c release from mitochondria, suggesting activation of the intrinsic apoptotic pathway. Tmz treatment also increased caspase-3 activation without changing caspase-8 levels compared with TRAIL alone, consistent with intrinsic pathway activation (35). Several other reports have suggested that RT augments TRAIL-based therapies by various mechanisms, including upregulation of DR5, Bak, and Bax and inhibition of bcl-2 (36–38). RT induced

p53-dependent upregulation of DR5 in breast cancer cell lines (39), and increased Bax expression enhanced TRA-8 apoptosis in glioma cell lines (40). RT and TRAIL were synergistic in a lymphoma cell line that overexpressed anti-apoptotic bcl-2 protein, but they were only additive in a cell line without bcl-2 overexpression (41). It is unclear whether RT will sensitize normal cells to TRAIL to any degree.

The clinical application of monoclonal antibodies such as TRA-8 in malignant glioma might be limited by antibody penetration into the tumor. Contrast-enhancing tumors such as glioblastoma multiforme typically have a disturbed blood–brain barrier such that macromolecules can cross in clinically relevant concentrations. If systemically administered antibodies do not localize in the tumor radiographically, another option is local administration with convective enhanced delivery or intratumoral injection. Intracranial animal studies of TRAIL administered with convective enhanced delivery have demonstrated enhanced survival in combination with Tmz (35), such as we have observed with systemic administration of TRA-8. Challenges to the development of TRA-8 and other TRAIL-based therapies for gliomas include tumor delivery, optimal timing with chemotherapy agents, and RT, and molecular identification of resistant tumors that might benefit from combination with other targeted therapies. Future work will extend this subcutaneous model to other malignant glioma xenografts and expand the intracranial model to improve statistical power.

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