

The combination of novel low molecular weight inhibitors of RAF (LBT613) and target of rapamycin (RAD001) decreases glioma proliferation and invasion

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Abstract

Monotherapies have proven largely ineffective for the treatment of glioblastomas, suggesting that increased patient benefit may be achieved by combining therapies. Two protumorigenic pathways known to be active in glioblastoma include RAS/RAF/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKT/target of rapamycin (TOR). We investigated the efficacy of a combination of novel low molecular weight inhibitors LBT613 and RAD001 (everolimus), which were designed to target RAF and TOR, respectively. LBT613 decreased phosphorylation of extracellular signal-regulated kinase 1 and 2, downstream effectors of RAF, in a human glioma cell line. RAD001 resulted in decreased phosphorylation of the TOR effector S6. To determine if targeting RAF and TOR activities could result in decreased protumorigenic glioma cellular behaviors, we evaluated the abilities of LBT613 and RAD001 to affect the proliferation, migration, and invasion of human glioma cells. Treatment with either LBT613 or RAD001 alone significantly decreased the proliferation of multiple human glioma cell lines. Furthermore, LBT613 and RAD001 in combination synergized to

decrease glioma cell proliferation in association with G₁ cell cycle arrest. Glioma invasion is a critical contributor to tumor malignancy. The combination of LBT613 and RAD001 inhibited the invasion of human glioma cells through Matrigel to a greater degree than treatment with either drug alone. These data suggest that the combination of LBT613 and RAD001 reduces glioma cell proliferation and invasion and support examination of the combination of RAF and TOR inhibitors for the treatment of human glioblastoma patients. [Mol Cancer Ther 2007;6(9):2449–57]

Introduction

Despite advances in the ability to diagnose and treat many cancers, successful treatment of primary brain tumors remains extremely challenging. Even with the most advanced therapies, the median survival of patients with glioblastoma multiforme (WHO grade IV glioma) has reached only 14.6 months (1). This limited survival for glioblastoma patients indicates a need for innovative therapies targeting glioblastomas. Novel therapies are likely to arise from the targeting of multiple molecular pathways altered during glioma formation and progression.

Two signal transduction cascades known to contribute to the formation of glioblastomas are the RAS/RAF/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT/target of rapamycin (TOR) pathways. Both RAS (2) and AKT (3, 4) activities are elevated in glioblastoma patient specimens. Activation of AKT and RAS promotes tumor progression by transducing signals from growth factor receptors to regulate multiple protumorigenic cellular behaviors, including proliferation, survival, and invasion (2, 5–12). Indeed, AKT activation is associated with the conversion of human glioma cells from anaplastic astrocytoma to glioblastoma in a cell culture model (13), and mutations of the AKT inhibitor phosphatase and tensin homologue are observed in glioblastomas but not in low-grade gliomas (14).

Cooperation of the RAS/RAF/MAPK and PI3K/AKT/TOR signal transduction pathways exists during brain tumor formation. Simultaneous enhancement of phosphorylation of MAPK, S6 kinase, and TOR—shared components of these signaling pathways—is associated with poor patient outcome (15). Additionally, a murine brain tumor model shows that the combination of RAS and AKT activation induces tumors in mice, but neither RAS nor AKT alone is sufficient to cause glioblastoma formation (4). This promotion of tumor formation by RAS and AKT is likely due to the combined enhancement of translation of distinct mRNAs (16) to affect multiple protumorigenic cellular behaviors.

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The cooperation of RAS/RAF/MAPK and PI3K/AKT/TOR signaling pathways during brain tumor formation suggests that the dual targeting of these signal transduction cascades may be beneficial for patient therapies. Previous results from our laboratory indicate that targeting RAF or TOR activities alone, with the small-molecule inhibitors AAL881 (17) and RAD001 (18), respectively, decreased glioma growth. Other researchers have determined that inhibiting AKT activity with the small-molecule inhibitors KP-372-1 and KP-372-2 decreased glioma cell proliferation due to increased apoptosis (6). A PI3K and TOR inhibitor, PI-103, was also shown to decrease glioma proliferation (19), showing that targeting the PI3K/AKT/TOR pathway at multiple steps inhibits glioma growth. Targeting integrin-linked kinase, a known activator of AKT, in combination with the RAF inhibitor GW5074 also showed a synergistic reduction in the colony-forming ability of glioma cells, which was associated with an increase in apoptosis (20). Based on these data and the known interaction between RAS/RAF/MAPK and PI3K/AKT/TOR signaling, we targeted both RAS and PI3K signaling using a unique combination of small-molecule inhibitors. We combined LBT613, an ATP-mimetic inhibitor of the kinase activity of RAF and vascular endothelial growth factor receptor 2, with the mammalian TOR inhibitor RAD001. We find that the combination of LBT613 and RAD001 significantly inhibits glioma cell proliferation more than either monotherapy. LBT613 and RAD001 synergize to decrease glioma growth through a mechanism associated with arrest of cells in the G₁ phase of the cell cycle. LBT613 and RAD001 also cooperate to reduce glioma cell migration and invasion, indicating that the dual targeting of RAS and PI3K signaling has multiple antitumorigenic effects on glioma cell behaviors.

Materials and Methods

Cell Lines and Culture

The human malignant glioma cell lines U87MG and U373MG were purchased from the American Type Culture Collection. U251MG was provided by Dr. Jan Poten (University of Uppsala, Uppsala, Sweden). DNA fingerprinting was done to eliminate the possibility of cross-contamination of U373MG and U251MG cells. D54MG is the Duke University subline of A-172. D54MG, U251MG, U87MG, and U373MG human glioma cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich).

Small-Molecule Inhibitors

LBT613 and RAD001 were generously provided by Novartis Institutes for Biomedical Research (Cambridge, MA). Each agent was dissolved in DMSO (Sigma-Aldrich) at a concentration of 10 mmol/L and stored at -80°C . For all experiments, LBT613 and/or RAD001 1000 \times stocks were added directly to the medium of treated cells to produce the indicated final concentrations of inhibitors.

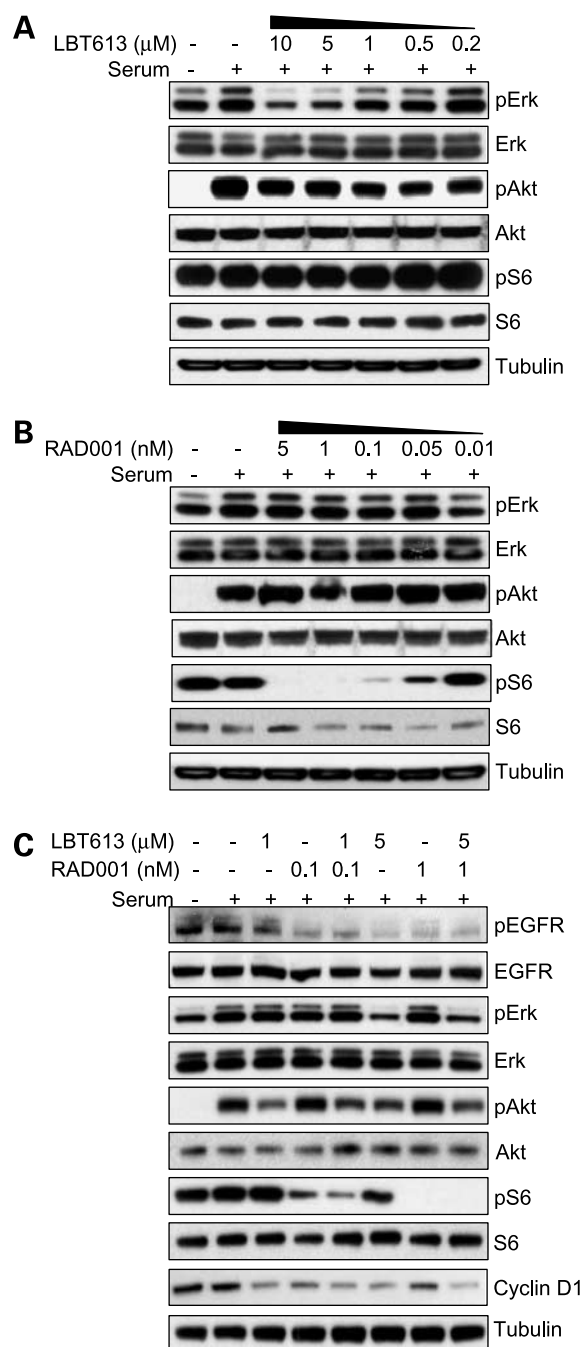


Figure 1. LBT613 and RAD001 differentially target signal transduction. D54MG cells were serum starved overnight and then pretreated for 2 h with the indicated final concentration of drug or DMSO as a control. Cells were then treated with or without serum for 15 min and lysed. Total cell extracts were analyzed by Western blot with the indicated antibodies. **A**, LBT613 decreased ERK phosphorylation. LBT613 was designed to target B-RAF and C-RAF activity, so phosphorylation of a protein downstream of RAF, ERK, was assessed. *pErk*, phosphorylated ERK; *pAkt*, phosphorylated AKT; *pS6*, phosphorylated S6. **B**, RAD001 decreased S6 ribosomal protein phosphorylation. RAD001 was designed to target TOR, so phosphorylation of a protein downstream of TOR, S6 ribosomal protein, was assessed. **C**, the combination of LBT613 and RAD001 decreased both ERK and S6 ribosomal protein phosphorylation. *pEGFR*, phosphorylated epidermal growth factor receptor.

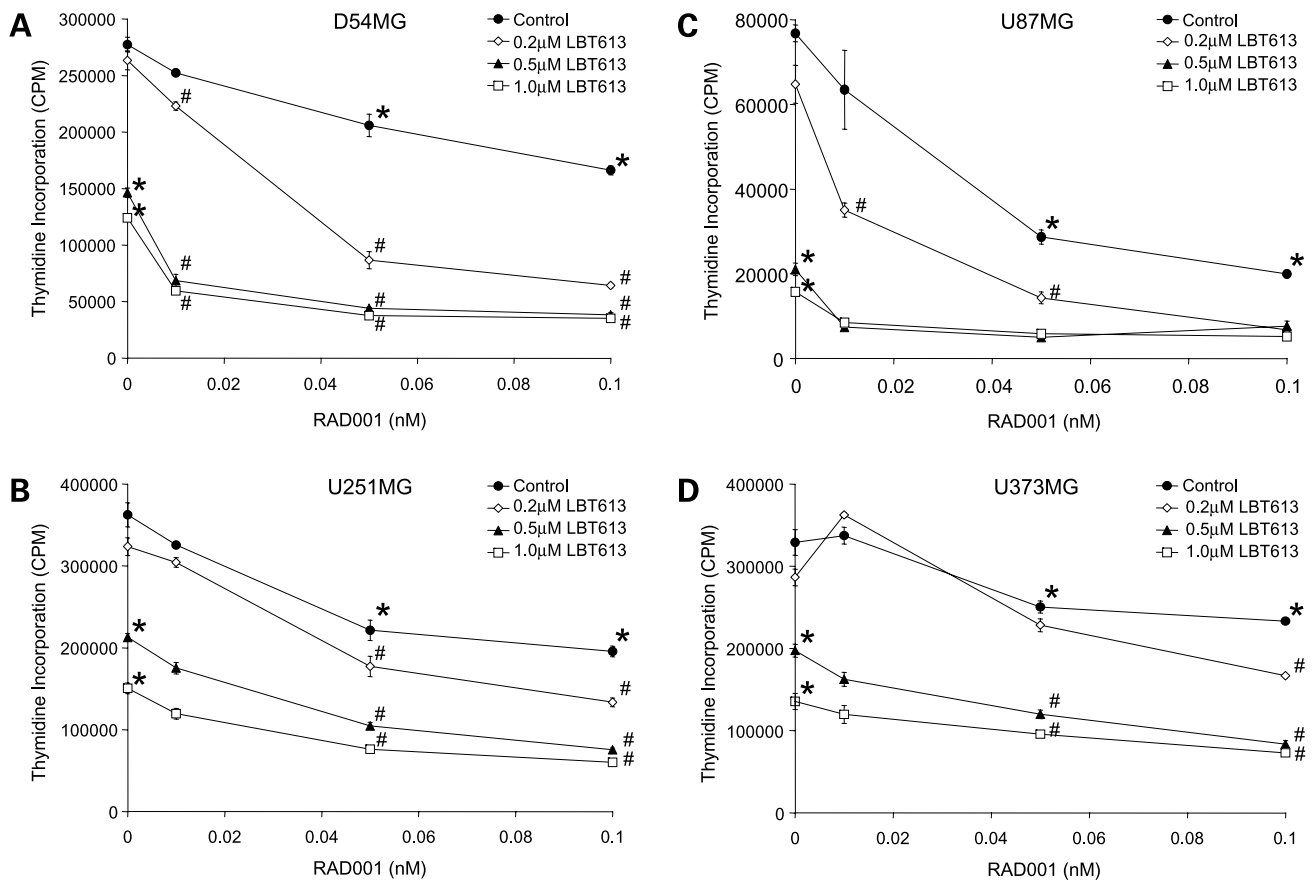


Figure 2. The combination of LBT613 and RAD001 significantly decreases proliferation more than either monotherapy. The human glioma cell lines D54MG (A), U251MG (B), U87MG (C), and U373MG (D) were serum starved overnight and then stimulated with serum in the presence of the indicated final concentration of drug(s) or DMSO as a control. After 44 h, [3 H]thymidine was added for 4 h and thymidine incorporation was determined as a measure of cell proliferation. *, $P < 0.001$ by ANOVA compared with control; #, $P < 0.05$ by ANOVA compared with LBT613 and RAD001 alone.

Western Blotting and Antibodies

Cells were serum starved overnight, pretreated with the indicated concentrations of drug or DMSO control for 2 h, and treated with serum (final 10%) or medium without serum for 15 min. Cells were then lysed in buffer [62.5 mmol/L Tris-HCl, 2% (w/v) SDS, 10% glycerol, 40 mmol/L DTT, protease inhibitors]. Protein content was determined using Bio-Rad Protein Assay Reagent. Equal protein amounts were run on SDS-polyacrylamide gels (Invitrogen), transferred to polyvinylidene difluoride membranes (Millipore), and probed with phosphorylated AKT, total AKT, phosphorylated S6, total S6, cyclin D1 (Cell Signaling Technology), phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (Promega), total ERK1, total ERK2, phosphorylated epidermal growth factor receptor, total epidermal growth factor receptor (Santa Cruz Biotechnology), and α -tubulin (Sigma-Aldrich). Proteins were detected using an enhanced chemiluminescence system (Pierce Biotechnology).

Thymidine Incorporation Assay

Cells were plated into 12-well plates at a density of 2×10^4 per well, serum starved overnight, and treated

with DMSO or increasing concentrations of RAD001 and/or LBT613 for 44 h in 10% serum. Cells were labeled for 4 h with 4 μ Ci [3 H]thymidine, fixed in 10% trichloroacetic acid, and lysed in 0.2 N NaOH. [3 H]thymidine incorporation into DNA was measured with a scintillation counter.

Combinatorial Index Calculation

Thymidine incorporation data were analyzed using CalcuSyn software (Biosoft) to generate a combination index (CI). A CI greater than one indicates antagonism, a CI of one indicates an additive effect, and a CI less than one indicates synergism.

Flow Cytometric and Annexin V Analysis

For cell cycle analysis, cells were plated into 10-cm dishes at a density of 3×10^5 per plate, serum starved overnight, and then treated for 48 h with DMSO control, 1 μ mol/L LBT613, 0.1 nmol/L RAD001, or 1 μ mol/L LBT613 and 0.1 nmol/L RAD001. Cells undergoing cell death become detached so conditioned media were collected. Treated cells were trypsinized and added to the conditioned medium. Cells were collected by centrifugation, washed with Dulbecco's PBS (Invitrogen), fixed in ethanol, and stained with propidium iodide. For Annexin V staining,

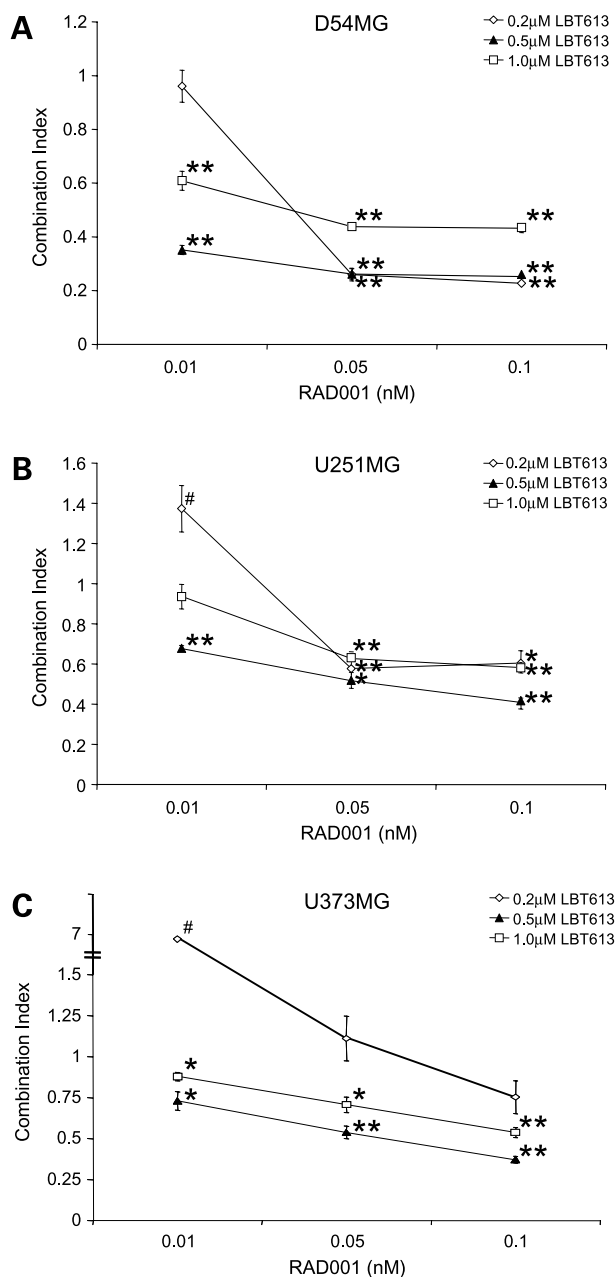


Figure 3. LBT613 and RAD001 synergize to reduce glioma proliferation. CalcuSyn software was used to determine the CI for the effect of the combination of the indicated concentrations of LBT613 and RAD001 on thymidine incorporation for D54MG (A), U251MG (B), and U373MG (C). For all cell lines, the calculated CI for multiple concentration combinations was significantly less than one, indicating synergism between LBT613 and RAD001. *, $P < 0.01$; **, $P < 0.001$, with one sample t test compared with a hypothetical mean of 1. Antagonism was only observed at one pair of concentrations (0.2 $\mu\text{mol/L}$ LBT613 and 0.01 nmol/L RAD001) in U251MG and U373MG. #, $P < 0.05$ by one sample t test compared with a hypothetical mean of 1.

cells were plated into six-well plates at a density of 1.5×10^5 per well and then treated for 48 h with DMSO control, 1 $\mu\text{mol/L}$ LBT613, 0.1 nmol/L RAD001, or 1 $\mu\text{mol/L}$ LBT613 and 0.1 nmol/L RAD001. Condition medium and trypsi-

nized cells were collected, washed with PBS, and incubated with Annexin V stain and propidium iodide as per the manufacturer's instructions (EMD Chemicals). For both cell cycle and apoptosis, analysis was done on FACScan gated to exclude cellular debris and collecting 10^4 events. Calculations were done using BD software.

Migration/Invasion Assays

Kits were purchased from BD Biosciences and used according to the manufacturer's instructions. Cells were serum starved for 48 h and treated with DMSO control or 1 $\mu\text{L/mL}$ of 1000 \times RAD001 and/or LBT613 dissolved in DMSO to produce indicated final concentrations of 1 $\mu\text{mol/L}$ LBT613, 1 nmol/L RAD001, or 1 $\mu\text{mol/L}$ LBT613 and 1 nmol/L RAD001. Treated cells were trypsinized, centrifuged, counted, and resuspended in serum-free medium to a concentration of 100,000 cells/mL. Cells were treated with DMSO control or indicated concentrations of RAD001 and/or LBT613, and 500 μL (50,000 cells) were placed in the upper Transwell chambers of inserts uncoated (migration) or coated with Matrigel (invasion). Bottom Transwell chambers were filled with 500 μL medium containing 10% fetal bovine serum as a chemoattractant. After 48 h, inserts were fixed and stained with Diff-Quick Fixative Solutions (Dade Behring, Inc.). Attached cells were imaged with a digital camera mounted to a light microscope (Olympus CK40) and quantified using ImageJ software.⁸ Experiments were done in triplicate. Cell numbers were assessed using ImageJ software by converting RGB color images to 8-bit black and white images, using the threshold function to designate the black pixels (cells) to be counted, and then using the analyze particles function to ascertain the average pixel area covered in each image.

Statistical Analysis

Significance was determined using GraphPad InStat 3 Software (GraphPad Software, Inc.) with ANOVA analysis except for CI statistics where the one sample t test was used to compare the mean to a hypothetical mean of 1.

Results

LBT613 and RAD001 Effectively Target Different Signal Transduction Pathways in Human Glioma Cells

To validate the abilities of RAD001 and LBT613 to effectively target the RAF and TOR signal transduction pathways, respectively, we examined the phosphorylation of downstream components of each pathway, ERK for RAF signaling and S6 ribosomal protein for TOR signaling, as well as AKT due to the potential for loss of upstream inhibitory feedback loops in the TOR pathway (21). The efficacy of both drugs was evaluated in D54MG, a human glioma cell line previously shown in our laboratory to express B-RAF and C-RAF proteins without mutations (17). AKT signaling is active in D54MG cultures because, like many human gliomas, these cells express a nonfunctional mutant form of phosphatase and tensin homologue, which

⁸ <http://rsb.info.nih.gov/ij/>

does not inhibit the PI3K/AKT pathway (data not shown). As expected, serum induced the phosphorylation of ERK and AKT in serum-starved D54MG cells (Fig. 1). Treatment with LBT613 decreased phosphorylation of ERK1 and

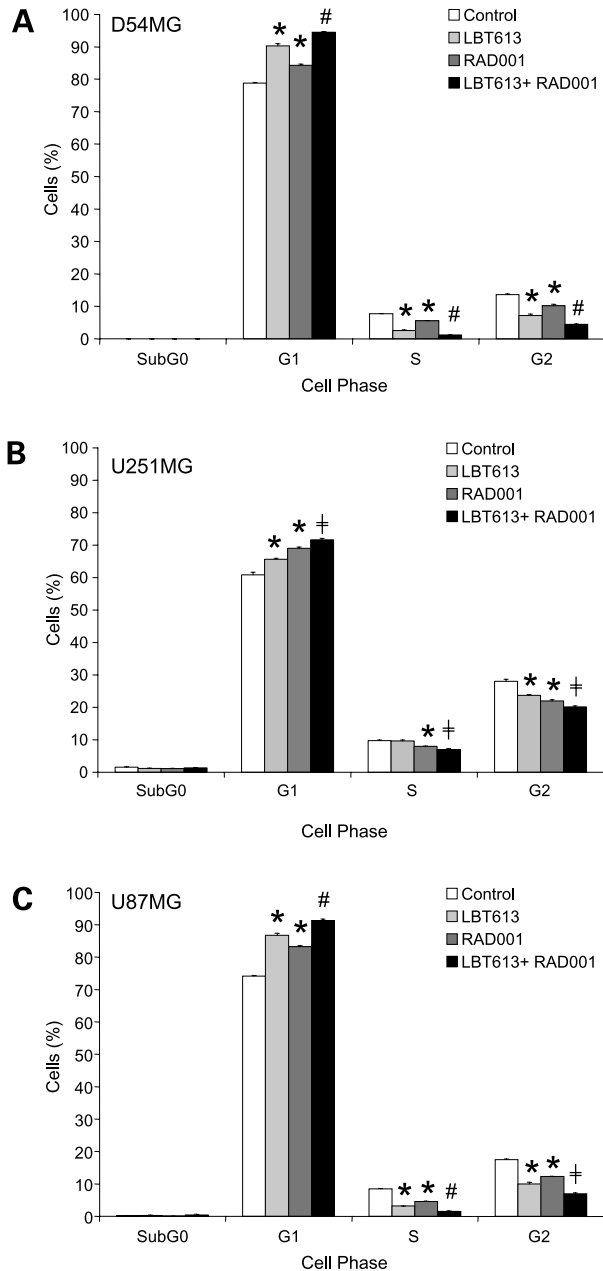


Figure 4. The combination of LBT613 and RAD001 significantly increases the proportion of cells in G₁ arrest more than either monotherapy. The human glioma cell lines D54MG (A), U251MG (B), and U87MG (C) were serum starved overnight and then stimulated with serum in the presence of 1 μ mol/L LBT613, 0.1 nmol/L RAD001, 1 μ mol/L LBT613 and 0.1 nmol/L RAD001, or DMSO control. After 48 h, nonadherent and attached cells were harvested, stained with propidium iodide, and analyzed by flow cytometry. *, $P < 0.001$ by ANOVA compared with control; #, $P < 0.001$ by ANOVA compared with LBT613 and RAD001 alone; †, $P < 0.05$ by ANOVA compared with LBT613 and RAD001 alone.

ERK2 in a concentration-dependent manner with 5 μ mol/L LBT613 sufficient to decrease ERK phosphorylation to baseline (Fig. 1A and C). This decrease in ERK phosphorylation was not due to a decrease in total ERK levels (Fig. 1A and C). LBT613 did not affect the phosphorylation or total levels of S6 ribosomal protein but decreased AKT phosphorylation without affecting total AKT levels (Fig. 1A and C). These effects of LBT613 on downstream signal transduction were consistent with the effects of other RAF kinase small-molecule inhibitors, such as AAL881 (17).

In contrast to the effects of LBT613, RAD001 treatment did not alter the phosphorylation or total levels of ERK1, ERK2, or AKT (Fig. 1B and C). RAD001 decreased the activating phosphorylation of the downstream S6 ribosomal protein in a concentration-dependent manner with 0.1 nmol/L sufficient to achieve complete inhibition (Fig. 1B and C). This decrease in phosphorylation was not due to a decrease in total protein levels as both total S6 ribosomal protein and tubulin control were not affected by RAD001 treatment (Fig. 1B and C).

The combination of LBT613 and RAD001 attenuated the activating phosphorylation of ERK1, ERK2, AKT, and S6 ribosomal protein without modulating total protein levels (Fig. 1C). The decrease in target protein phosphorylation was not enhanced by the combination of LBT613 and RAD001, but the use of both small-molecule inhibitors does simultaneously targeted RAF and TOR signal transduction with 1-h pretreatment (Fig. 1C) and may be beneficial long term (Supplementary Fig. S1).⁹ We also found that the combination of inhibitors blocked the autocrine loop for activation of the epidermal growth factor receptor, suggesting that this combination of inhibitors may provide additional benefit by inhibiting epidermal growth factor receptor signaling (Fig. 1C). Therefore, these data show that LBT613 and RAD001 can be used together in human glioma cells to effectively target distinct signal transduction pathways and can have added benefit through combinatorial use.

The Combination of LBT613 and RAD001 Synergistically Decreases Glioma Cell Proliferation

To determine if the simultaneous targeting of RAF and TOR signal transduction could decrease protumorigenic glioma cell behaviors, we first determined the effects of LBT613, RAD001, or the combination of these inhibitors on human glioma cell proliferation. The proliferation of D54MG, U251MG, U87MG, and U373MG human glioma cells was decreased in a concentration-dependent manner by treatment with either LBT613 or RAD001 (Fig. 2). LBT613 (0.5 μ mol/L) or RAD001 (0.05 nmol/L) was sufficient to significantly decrease glioma cell proliferation, as measured by thymidine incorporation, in all of the cell lines studied (Fig. 2; *, $P < 0.001$ by ANOVA compared with control). Every glioma cell line tested also proliferated less when LBT613 and RAD001 were used in combination

⁹ Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

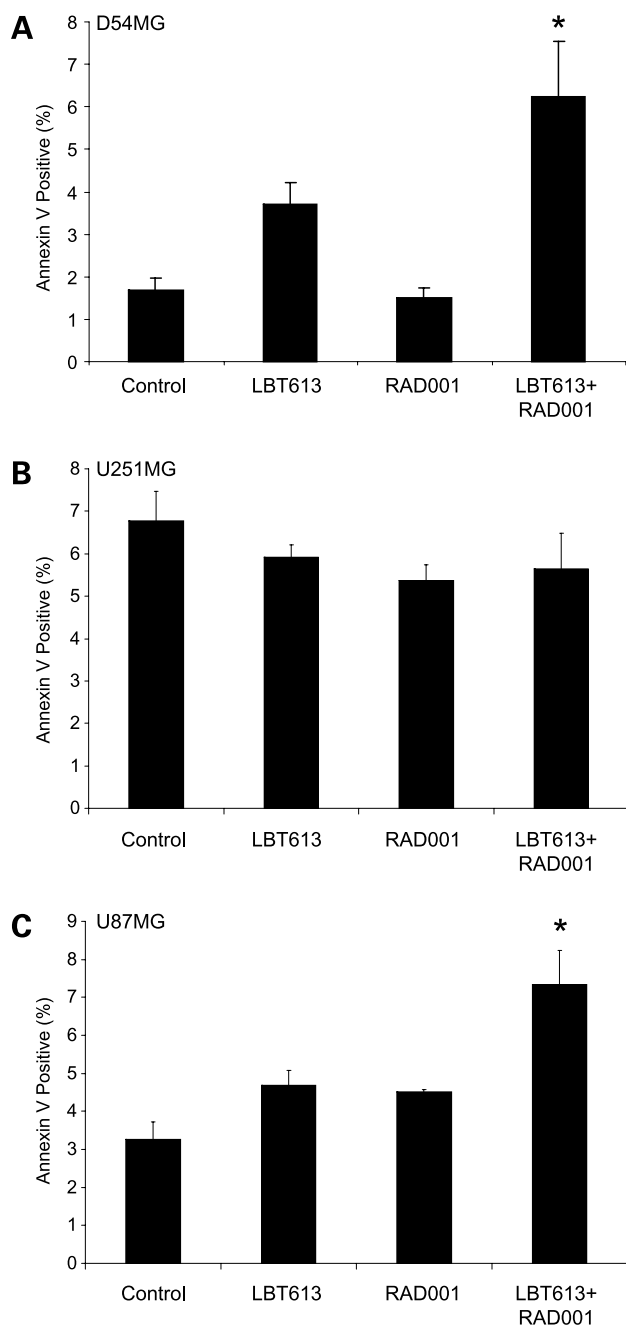


Figure 5. The combination of LBT613 and RAD001 significantly increases apoptosis in a cell line-dependent manner. The human glioma cell lines D54MG (A), U251MG (B), and U87MG (C) were treated with 1 $\mu\text{mol/L}$ LBT613, 1 nmol/L RAD001, 1 $\mu\text{mol/L}$ LBT613 and 1 nmol/L RAD001, or DMSO control. After 48 h, nonadherent and attached cells were harvested, incubated with Annexin V-FITC antibody in the presence of propidium iodide, and analyzed by flow cytometry. The percentage of all Annexin V-positive cells is displayed. *, $P < 0.01$ by ANOVA compared with control.

compared with either monotherapy alone (Fig. 2; *, $P < 0.05$ by ANOVA compared with either LBT613 or RAD001). However, the concentrations necessary to achieve a combinatorial benefit differed slightly between cell lines.

The minimal concentration of the combined inhibitors necessary to inhibit proliferation more than either drug alone was 0.2 $\mu\text{mol/L}$ LBT613 and 0.01 nmol/L RAD001 for D54MG (Fig. 2A) and U87MG cells (Fig. 2C), 0.2 $\mu\text{mol/L}$ LBT613 and 0.05 nmol/L RAD001 for U251MG cells (Fig. 2B), and 0.2 $\mu\text{mol/L}$ LBT613 and 0.1 nmol/L RAD001 for U373MG cells (Fig. 2D). These data show that, although there are differences in the sensitivity of glioma cells to the combination of LBT613 and RAD001, LBT613 and RAD001 cooperate to reduce the growth of multiple human glioma cell lines.

To determine if the cooperation between LBT613 and RAD001 was additive or synergistic, the CI for each combination of drug concentrations was calculated using CalcuSyn software. We found that, in D54MG (Fig. 3A), U251MG (Fig. 3B), and U373MG (Fig. 3C), LBT613 and RAD001 synergized to reduce cell proliferation as indicated by the ability of multiple drug concentration combinations to produce a CI of less than one. The lowest CI, or greatest synergy, was produced with the combination of 0.2 $\mu\text{mol/L}$ LBT613 and 0.1 nmol/L RAD001 in D54MG (CI = 0.22; Fig. 3A) and with 0.5 $\mu\text{mol/L}$ LBT613 and 0.1 nmol/L RAD001 in U251MG (CI = 0.41; Fig. 3B) and U373MG (CI = 0.37; Fig. 3C). Therefore, LBT613 and RAD001 synergize to reduce the proliferation of human glioma cells.

The Combination of LBT613 and RAD001 Significantly Increases the Proportion of Cells in G₁ Arrest More than Either Monotherapy

To determine the mechanism by which glioma cell proliferation was inhibited by LBT613 and RAD001, we compared the cell cycle of control and treated cells. LBT613 (1 $\mu\text{mol/L}$) or RAD001 (1 nmol/L) significantly increased the percentage of D54MG (Fig. 4A), U251MG (Fig. 4B), and U87MG (Fig. 4C) cells in the G₁ phase of the cell cycle (*, $P < 0.001$ by ANOVA compared with control). The combination of 1 $\mu\text{mol/L}$ LBT613 and 1 nmol/L RAD001 further increased the percentage of cells in the G₁ phase of the cell cycle (*, $P < 0.05$; #, $P < 0.001$ by ANOVA compared with LBT613 or RAD001 alone). The increase in G₁ cell cycle arrest on LBT613 and RAD001 resulted in a significant decrease in the percentage of cells in the S and G₂ (Fig. 4A–C) phases of the cell cycle and may be due, in part, to a decrease in expression of cyclin D₁ (Fig. 1C). Thus, LBT613 and RAD001 reduced glioma cell proliferation by inducing a G₁ cell cycle arrest that prevents continuation through the cell cycle.

In the cell cycle analysis, we also observed no significant change in the sub-G₀ fraction of cells (Fig. 4) with LBT613 and RAD001 treatment, indicating there was no increase in the percentage of dead cells as detected with propidium iodide alone. To more fully determine if LBT613 and RAD001 could increase apoptosis, we examined the percentage of Annexin V-positive cells with monotherapy and the drug combination. Apoptosis increased with the combination of LBT613 and RAD001 compared with control in D54MG and U87MG (Fig. 5A and C; *, $P < 0.01$), whereas there was no effect in U251MG (Fig. 5B; $P = 0.42$). These data indicate that, although there is an

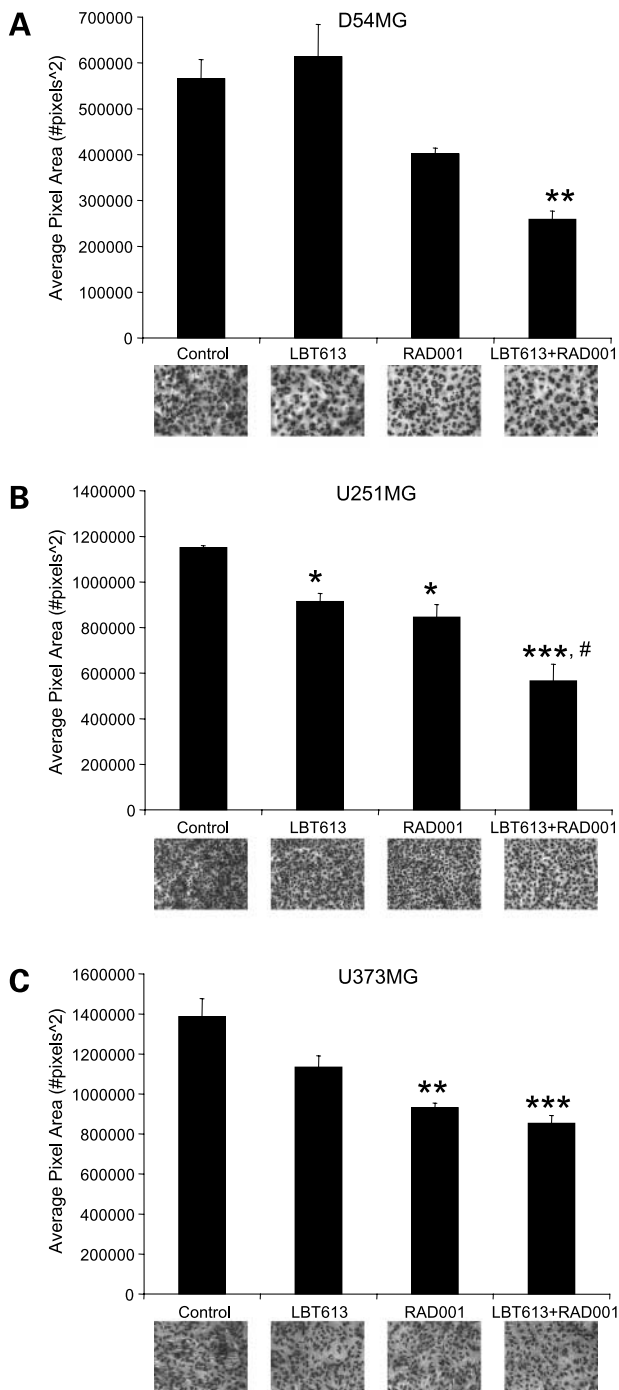


Figure 6. The combination of LBT613 and RAD001 significantly decreases migration. The human glioma cell lines D54MG (A), U251MG (B), and U373MG (C) were serum starved in the presence of 1 $\mu\text{mol/L}$ LBT613, 1 nmol/L RAD001, 1 $\mu\text{mol/L}$ LBT613 and 1 nmol/L RAD001, or DMSO control. After 48 h, cells were harvested and placed in serum-free medium in the upper chamber of an uncoated insert and allowed to migrate toward medium containing 10% fetal bovine serum. After 48 h, migrating cells were stained and quantified using the average pixel area covered in pictures of each well. *, $P < 0.05$ by ANOVA compared with control; **, $P < 0.01$ by ANOVA compared with control; ***, $P < 0.001$ by ANOVA compared with control; #, $P < 0.05$ by ANOVA compared with LBT613 and RAD001 alone.

increase in G_1 arrest and a decrease in proliferation with the combination of LBT613 and RAD001 across all glioma cell lines tested, there was not a similar broad increase in apoptosis at an identical concentration and time course of inhibitor treatment.

The Combination of LBT613 and RAD001 Significantly Decreases Glioma Migration and Invasion

To further investigate the effect of targeting RAF and TOR signals on protumorigenic glioma cell behaviors, we measured the effect of LBT613 and RAD001 on glioma cellular migration and invasion. In Boyden chamber assays, the combination of 1 $\mu\text{mol/L}$ LBT613 and 1 nmol/L RAD001 inhibited migration in D54MG (Fig. 6A), U251MG (Fig. 6B), and U373MG (Fig. 6C) cells (**, $P < 0.01$; ***, $P < 0.001$ by ANOVA compared with control). The combination of LBT613 and RAD001 inhibited invasion in all of the cell lines tested (Fig. 7; *, $P < 0.05$; ***, $P < 0.001$ by ANOVA compared with control). The decrease in glioma cell migration with LBT613 and RAD001 was significantly different from either monotherapy alone in U251MG, one of the three glioma cell lines tested (Fig. 6B; #, $P < 0.05$ by ANOVA compared with LBT613 or RAD001). However, the combination decreased invasion more than either monotherapy in D54MG and U373MG, two of the three glioma cell lines tested (Fig. 7A and C; #, $P < 0.05$ by ANOVA compared with LBT613 or RAD001). These data showed cell line-specific differences in the extent of the response to LBT613 and RAD001 treatment compared with either monotherapy. However, all of the human glioma cell lines displayed decreased migration and invasion on treatment with the combination of LBT613 and RAD001, showing that the combinatorial therapy is likely to be beneficial for reducing glioma cell movement.

Discussion

As our understanding of the molecular mechanisms involved in brain tumor formation increases, so will our potential avenues for therapeutic intervention. For example, elucidation of the cooperation between RAS and PI3K signaling in the formation of glioblastoma (4, 16) suggests that dual targeting of these signal transduction cascades could be beneficial for patient therapy. Therefore, we determined the efficacy of combining inhibitors of RAF and TOR, proteins downstream of RAS and PI3K, respectively, on glioma growth and invasion.

The proliferation of each glioma cell line tested synergistically decreased with the combination of the RAF inhibitor LBT613 and the TOR inhibitor RAD001, and this decrease in growth was associated with a G_1 cell cycle arrest. However, identical treatments with LBT613 and RAD001 did not produce a consistent increase in the percentage of apoptotic cells. Although the combination of LBT613 and RAD001 induced apoptosis in D54MG and U87MG cells, no apoptotic effect was observed in U251MG. When compared with the status of major tumor suppressor genes commonly altered in glioma, these data may indicate that loss of p53 mediates resistance to apoptosis induced

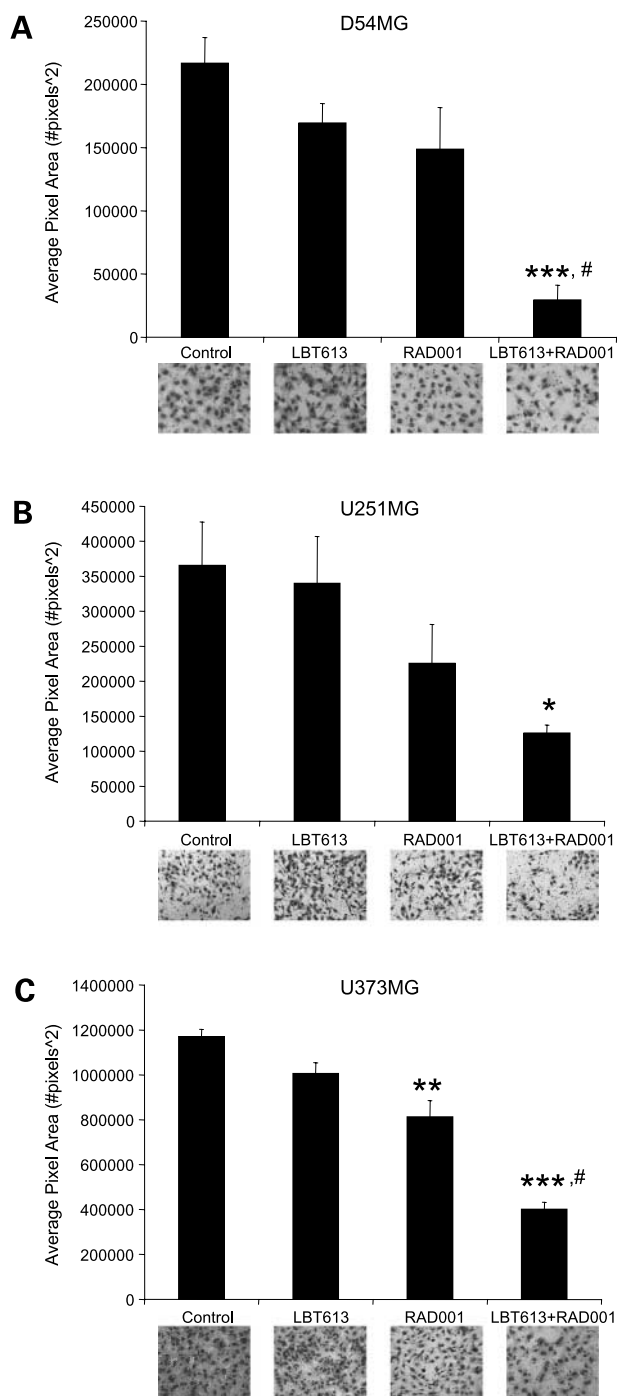


Figure 7. The combination of LBT613 and RAD001 significantly decreases invasion through an extracellular matrix. The human glioma cell lines D54MG (A), U251MG (B), and U373MG (C) were serum starved in the presence of 1 $\mu\text{mol/L}$ LBT613, 1 nmol/L RAD001, 1 $\mu\text{mol/L}$ LBT613 and 1 nmol/L RAD001, or DMSO control. After 48 h, cells were harvested and placed in serum-free medium in the upper chamber of a Matrigel-coated insert and allow to migrate toward medium containing 10% fetal bovine serum. After 48 h, invading cells were stained and quantified using the average pixel area covered in pictures of each well. *, $P < 0.05$ by ANOVA compared with control; **, $P < 0.01$ by ANOVA compared with control; ***, $P < 0.001$ by ANOVA compared with control; #, $P < 0.05$ by ANOVA compared with LBT613 and RAD001 monotherapies.

by the combination of LBT613 and RAD001. D54MG and U87MG are p53 wild-type, whereas U251MG expresses a mutant form of p53. All of the cell lines have mutated or deleted phosphatase and tensin homologue and the cyclin-dependent kinase inhibitor p16INK4A. Thus, variations in p53 status, but not phosphatase and tensin homologue or p16INK4A, could help to explain the cell type-specific differences in apoptosis in response to LBT613 and RAD001 treatment. This variability in cell survival also likely indicates that tumor responses may differ depending on the genetic mutations, which have accumulated during glioma development and progression.

In addition to its effects on cell growth and survival, the combination of LBT613 and RAD001 decreases glioma cell migration and invasion, suggesting an added benefit of the combination of RAF and TOR inhibitors for the treatment of gliomas. Indeed, the ability to prevent glioma cell invasion and target infiltrative glioma cell growth would represent a potential advance in our ability to treat brain tumors. Unlike most other types of cancer, the morbidity and mortality from glioblastomas comes from local invasion rather than metastasis. This local invasion prevents complete surgical resection as shown by the recurrence of the majority of glioblastomas (80–90%) within 2 cm of the original tumor site (22, 23). As glioma cells frequently extend through much of the neural axis before diagnosis, even local control of invasion will unfortunately not be curative. Therefore, novel therapies targeting the infiltrative glioma cells must be able to act some distance from the surgical site on cells that are disseminated into normal brain. Although it remains to be determined if the combination of RAF and TOR inhibitors could be an anti-invasive therapy, our *in vitro* data do show that this combination could have benefits for patient therapies beyond a simple reduction of cancer cell proliferation.

Our data strongly suggest that the combination of RAF and TOR inhibitors could reduce glioma cell growth and invasion and that further development of this novel therapeutic paradigm is warranted, including examination of the effects of LBT613 and RAD001 *in vivo*. Although quantities of these novel compounds are limited, we were able to do a preclinical animal trial of LBT613 and RAD001 in a D54MG xenograft model. Although there were two deaths in a total of 10 animals attributed to toxicity, treatment of immunocompromised mice with established tumors with 100 mg/kg LBT613 and 15 mg/kg RAD001 significantly delayed the growth of the tumors by 6.7 days ($P < 0.001$ by the Wilcoxon rank-sum test) as assessed by the average difference in days required for tumors to reach a five times the initial tumor volume. Although these *in vivo* data further support further examination of the use of LBT613 and RAD001 in combination for glioma therapy, it is too early to determine if the *in vitro* antitumorigenic effects we have observed across multiple cell lines will translate into a reduction in the growth of multiple xenografts *in vivo*. However, the use of LBT613 and RAD001 in combination to reduce glioma growth and

invasion seems promising, and our data suggest that glioma cell growth could be decreased by other combinations of available novel small-molecule inhibitors that target different components of the RAS/RAF/MAPK and PI3K/AKT/TOR pathways.

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