

Inhibition of PI3K/mTOR pathways in glioblastoma and implications for combination therapy with temozolomide

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Due to its molecular heterogeneity and infiltrative nature, glioblastoma multiforme (GBM) is notoriously resistant to traditional and experimental therapeutics. To overcome these hurdles, targeted agents have been combined with conventional therapy. We evaluated the preclinical potential of a novel, orally bioavailable PI3K/mTOR dual inhibitor (XL765) in *in vitro* and *in vivo* studies. *In vivo* serially passaged human GBM xenografts that are more genetically stable than GBM cell lines in culture were used for all experiments. Biochemical downstream changes were evaluated by immunoblot and cytotoxicity by colorimetric ATP-based assay. For *in vivo* experiments, human xenograft GBM 39 grown intracranially in nude mice was altered to express luciferase to monitor tumor burden by optical imaging. XL765 resulted in concentration-dependent decreases in cell viability *in vitro*. Cytotoxic doses resulted in specific inhibition of PI3K signaling. Combining XL765 with temozolomide (TMZ) resulted in additive toxicity in 4 of 5 xenografts. *In vivo*, XL765 administered by oral gavage resulted in greater than 12-fold reduction in median tumor bioluminescence compared with control (Mann–Whitney test $p = 0.001$) and improvement in median survival (logrank $p = 0.05$). TMZ alone showed a 30-fold decrease in median bioluminescence, but the combination XL765 + TMZ yielded a 140-fold reduction in median bioluminescence (Mann–Whitney test $p = 0.05$) with a trend toward improvement in median survival (logrank $p = 0.09$) compared with

TMZ alone. XL765 shows activity as monotherapy and in combination with conventional therapeutics in a range of genetically diverse GBM xenografts.

Keywords: PI3K/mTOR inhibitor, glioma, temozolomide, signaling inhibitor.

The current standard of care for glioblastoma multiforme (GBM) is postoperative radiation and temozolomide (TMZ), producing a median survival of approximately 14 months.¹ Despite extensive translational research and development of experimental therapeutics, there has been no significant improvement in overall survival for patients.² A significant hurdle is the molecular heterogeneity of GBM,^{3,4} which impedes uniform application of specific molecularly targeted agents. One frequently dysregulated pathway is the receptor tyrosine kinase (RTK)/phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) molecular cascade, which is activated by various mechanisms in GBM.⁵ Analysis of 209 GBM clinical samples by the Cancer Genome Atlas group demonstrated that 86% had a genetic alteration (activating mutation or gene amplification) in the RTK/PI3K pathway.⁶ The majority of these alterations occurred in the various RTK signaling inputs including *EGFR*, *ERBB2*, *PDGFRA*, and *MET*, while alterations in genes encoding subunits of PI3K itself (PIK3R1 and PIK3CA) were the second most common event. In addition, 36% of GBM samples had mutations or homozygous deletions of phosphatase and tensin homolog (PTEN), a tumor suppressor protein that regulates PI3K activity. Clinically, retrospective data indicate that genetic alterations in low-grade gliomas resulting

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in PI3K pathway activation have a detrimental impact on patient survival.³⁶

Attempts to inhibit the PI3K pathway with pan-PI3K inhibitors such as LY294002^{7,8} and wortmannin/PX-866,^{9,10} while successful preclinically, have not progressed to clinical use due to concerns over organ toxicity and poor bioavailability. Inhibition of the pathway distally using rapamycin resulted in paradoxical activation of Akt through loss of negative feedback in a subset of patients, which in turn was associated with shorter time-to-progression during postsurgical maintenance rapamycin therapy.¹¹ Development of next-generation, class I α -specific PI3K inhibitors with oral bioavailability has led to a resurgence in efforts to therapeutically modulate this pathway.¹² Identification of one particularly effective PI3K inhibitor also led to the discovery of its ability to concomitantly inhibit mTOR.¹³ Since then, additional studies have focused on dual inhibition of both PI3K and mTOR in GBM.^{14–18} However, it remains unclear which subtypes of GBM are susceptible to this approach. To help answer this question, we obtained a panel of genetically characterized human GBM xenografts serially passaged in nude mice to maintain genetic stability.^{19,20} We used the PI3K/mTOR inhibitor XL765, which has demonstrated broad anticancer efficacy.²¹

In addition to evaluating effects of XL765 *in vitro* against a genetically diverse panel of GBM xenografts, we combined XL765 with TMZ to determine the combined cytotoxic efficacy of these agents. In an intracranial, orthotopic xenograft glioma model that allows tumor burden to be monitored noninvasively, we assessed the efficacy of XL765 *in vivo* as a single agent and in combination with TMZ. This study provides a basis for clinical investigation of XL765 combined with TMZ in phase Ib/II trials.

Materials and Methods

Cell Culture

GBM 6, GBM 8, GBM 12, GBM 39, and GS-2 cell lines have been previously described.¹⁹ The identities of all cell sources used for these investigations were confirmed by short tandem repeat DNA fingerprinting using the Promega Powerplex 1.2 platform to determine the status of *EGFR*, *PTEN*, *p53*, *MGMT*, and *p16*. Xenografts were harvested from the flanks of nude mice, diced, filtered, and washed 3 times with fresh media. Cells were grown at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 μ g/mL penicillin, 100 units/mL streptomycin, nonessential amino acids, and 2 mM glutamine (UCSF Cell Culture facility). XL765 (Exelixis) stock was stored as a 10-mM solution in dimethyl sulfoxide (DMSO) at –20°C. Temozolomide (Schering-Plough) stock was stored as an 80-mM solution in DMSO at –20°C.

Cell Viability Assay

Glioblastoma cells were plated in 96-well black-walled plates at a density of 3000 cells per well. Cells were allowed to adhere for 16 hours. Medium was then removed and replaced with medium containing specified concentrations of drug(s). Viable cells were quantitated 72 hours after drug exposure using the CellTiter-Glo Assay (Promega) per manufacturer's instruction. Results are presented as the mean value \pm standard error from three independent experiments.

Immunoblot

Effects of XL765 on activity of PI3K and mTOR pathways were analyzed by immunoblot. Cells in 10-cm plastic dishes were treated with specified concentrations of XL765. After 24 hours, the cells were lysed by incubation in lysis buffer (50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 1% NP-40, 1.5 mM MgCl₂, and 10 mM EDTA) with proteinase inhibitor cocktail added (Roche Diagnostics Corporation) at 4°C. Protein lysate (20 μ g) was loaded and separated on 4–20% Tris-Glycine gels (Invitrogen) and transferred to pre-wetted polyvinylidene fluoride membranes. Membranes were blocked with 4% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 hour at room temperature. Membranes were then incubated with primary antibody overnight at 4°C. Blots were washed with TBST three times for 15 minutes each and then incubated for 1 hour at room temperature with secondary antibody. Bands were visualized using enhanced chemiluminescence detection reagent (Amersham Pharmacia). Antibodies against pAKT, pS6, p4EBP1, pPRAS40, pJNK, pp38, total AKT, total S6, total 4EBP1, and total PRAS40 were obtained from Cell Signaling Technology; anti- β -actin antibody from Sigma-Aldrich; and anti-rabbit and anti-mouse antibodies from Amersham Pharmacia.

Animal Model

Athymic 4- to 6-week-old nude-Foxn1^{nu} mice (Taconic Farms) were used for all *in vivo* experiments. All procedures were performed according to a protocol approved by the University of California – San Francisco (UCSF) Institutional Animal Care and Use Committee (IACUC). GBM 39 cells with luciferase gene modification were harvested from the flanks of nude mice. Cells were mechanically disaggregated, filtered, and washed 3 times with fresh media. Nude mice were manually injected with 3×10^5 cells intracranially in the right caudate-putamen using a 26G needle attached to a Hamilton syringe. Mice were randomized into groups of 10 animals per group based on bioluminescence on day 20 after injection, and treatments were started on day 21. Mice were treated as follows: (1) *Control*: 100 μ l of Ora-Plus (Paddock Laboratories)

by oral gavage once per day on days 21–25, 28–32, 49–53, and 56–60; (2) XL765: 100 μ l (50 mg/kg dissolved in 10 mM HCl) by oral gavage twice per day (6 hours apart) on days 21–25, 28–32, 49–53, and 56–60; and (3) TMZ: 100 μ l (5 mg/kg dissolved in Ora-Plus) once per day on days 21–25 and 49–53. The dose of XL765 (50 mg/kg twice a day) was chosen after higher doses (specifically 60 mg/kg twice a day and 100 mg once a day) resulted in progressive body weight loss and morbidity requiring euthanasia according to IACUC humane treatment guidelines. When XL765 and TMZ were given in combination, TMZ was given 30 minutes after XL765. Mouse weights were recorded daily. If an animal's weight dropped 10% below baseline, treatment was withheld until the weight recovered. Animals were sacrificed if they became symptomatic from intracranial tumor burden according to the IACUC protocol.

Bioluminescence Imaging

In vivo bioluminescence images were obtained using the IVIS Imaging System 100 series (Xenogen Corporation). Beginning on day 11 post-injection, mice were injected with 150 mg/kg i.p. D-luciferin. Thirteen minutes after injection, mice were anesthetized with isoflurane and imaged using various exposure times (ranging from 1 s to 2 m) to optimize images. Whole brain bioluminescence was measured for each mouse as well as mean bioluminescence for each treatment group. All mice were imaged 3 times per week, on Monday, Wednesday, and Friday. Previously published data using our model have documented a good correlation between measured bioluminescence, tumor burden, and benefit or detriment in animal survival.⁴⁰ For statistical analysis comparing treatment groups, bioluminescence values from the last day where all mice in both groups were alive were used.

Immunohistochemistry

Mice whose tumors were to be harvested for immunohistochemistry (IHC) were monitored with bioluminescence imaging until their relative radiance was between 5×10^5 and 1×10^6 , at which point they were treated by the appropriate agents (control, XL765, TMZ, or XL765 + TMZ) for 2 consecutive days and then sacrificed. Brains were harvested and frozen in optimum cutting temperature compound (Tissue Tek) and placed in -80°C . Brains were then sectioned (UCSF tissue core facility), stained with primary antibody against pS6 (UCSF IHC and molecular pathology core facility), and photographed and interpreted by Dr. Joanna Phillips (UCSF Department of Pathology). After relative quantification of pS6 staining, representative slides were documented.

Statistical Analysis

All statistical analyses were done under the supervision of the biostatistics division of the UCSF Brain Tumor

Research Center using the software program MedCalc. Data for cytotoxicity of XL765 at various concentrations were generated by regression analysis with fitting to a quadratic equation ($y = a + bx + cx^2$) with XL765 concentration as the independent variable. To determine whether the combination of XL765 + TMZ significantly differed from single agent treatment alone in vitro, the most effective single agent (XL765 or TMZ) was compared with the combination using a Wilcoxon rank-sum test. In vivo, tumor bioluminescence between groups was statistically compared using the Mann–Whitney test for independent samples. Survival was analyzed using Kaplan–Meier survival curves.

Results

XL765 Inhibits Activation of PI3 Kinase and mTOR Pathways

We sought to evaluate effects of XL765 treatment on the PI3K and mTOR pathways in vitro using a variety of human GBM xenografts (Table 1). These xenografts were harvested from the flanks of nude mice, grown in culture, and exposed to increasing concentrations of XL765 as a single agent. Immunoblots were done probing for phosphorylation of key molecules downstream of PI3K (pAkt and pPRAS40) and mTOR (pS6 and p4EBP1). In addition, phosphoproteins within other signaling cascades (pp38 and pJNK) were assessed to determine the specificity of XL765 and showed no changes in phosphorylation (data not shown). After exposure to XL765, cells were lysed and protein expression quantitated at various time points ranging from 1 to 36 hours, with 16 hours determined to be the optimal time point. At this time point, we were able to observe concentration-dependent inhibition of pAkt, pPRAS40, pS6, and p4EBP1 (Fig. 1), with β -actin used as a loading control. Levels of pAkt and pS6 were inhibited completely at concentrations of 2–8 μM , while pPRAS40 and p4EBP1 generally required 16 μM for similar levels of inhibition. Conversely, expression of nonphosphoprotein expression was not affected by XL765 in GBM 39, as shown in the last column of Figure 1.

Cytotoxic Activity of XL765 and TMZ in GBM Cells In Vitro

In order to determine the cytotoxicity of XL765 in GBM xenografts in vitro, we used a colorimetric ATP-based assay. All 5 GBM xenografts demonstrated a concentration-dependent decrease in cell viability after exposure to XL765 that was statistically significant based on quadratic regression analysis (Fig. 2). Based on the regression model, values of the half maximal inhibitory concentration for each xenograft were determined to be 7.5 μM for GBM 6, 5.7 μM for GBM 8, 3.7 μM for GBM 12, 7.7 μM for GBM GS-2, and 5.0 μM for GBM 39. Addition of TMZ to XL765 resulted in a statistically significant improvement in

Table 1. Genetic Profiles of Human GBM Xenografts Used in This Study

Xenograft	EGFR	PTEN	p53	p16	MGMT
GBM 6	Amplified (vIII)	Null	wt	Null	Nonhypermethylated
GBM 8	Amplified (wt)	Null	wt	Null	Hypermethylated
GBM 12	Amplified (wt)	wt	Null	Null	Hypermethylated
GBM GS-2	Nonamplified	Null	Mutant	Unknown	Unknown
GBM 39	Amplified (vIII)	wt	wt	Null	Hypermethylated

wt, wild type.

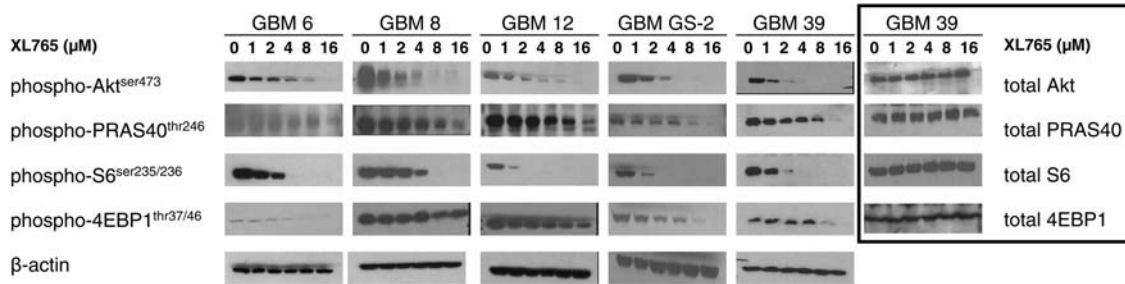


Fig. 1. XL765 results in downregulation of phosphorylated proteins downstream of PI3K (pAkt and pPRAS40) and mTOR (pS6 and p4EBP1) in human xenografts cultured in vitro. Phosphoprotein levels with β -actin loading controls are shown for GBM 6, GBM 8, GBM 12, GBM GS-2, and GBM 39. The same corresponding non-phosphoproteins are shown for GBM 39. Each experiment was done in duplicate with representative examples shown.

cytotoxicity in 4 out of 5 GBM xenografts, compared with the most cytotoxic single agent (Fig. 3).

Synergistic Activity of XL765 and TMZ In Vivo

To evaluate the effects of XL765 on tumor growth in vivo, we established tumors as intracranial xenografts in nude mice. GBM 39 cells had been modified to express a luciferase reporter in order to track tumor growth in vivo noninvasively. Mice were implanted intracranially with 5×10^5 GBM 39-*luc* cells. Twenty-one days post-implantation, intracranial tumor volumes were quantitated and mice were randomized into treatment groups. On day 42, the last day that all animals were alive, median bioluminescence values were 4.0×10^6 for control, 3.2×10^5 for XL765 (30 mg/kg), 1.2×10^5 for TMZ (5 mg/kg), and 2.9×10^4 for the combined treatment (Fig. 4). Therefore, on the last day that all animals were alive, the tumor burden was decreased compared with control by 13-fold in the XL765 group ($p = 0.001$), 34-fold in the TMZ group ($p = 0.0002$), and 140-fold in the XL765 + TMZ group ($p < 0.0001$). Compared with TMZ alone, the combination of XL765 + TMZ showed a 16-fold decrease in tumor burden ($p = 0.05$) at day 63 (the final day on which all mice in both groups were alive). In addition, IHC staining of tumor sections from these intracranial xenografts showed reduced pS6 expression in groups treated with XL765 (Fig. 4B).

To analyze survival times of the treatment groups, we generated Kaplan–Meier survival curves (Fig. 5). Median survival times were 55 days for control, 68

days for XL765 ($p = 0.05$, compared with control), 83 days for TMZ ($p = 0.001$), and 117 days for XL765 + TMZ ($p < 0.001$). In comparison with TMZ monotherapy, XL765 + TMZ demonstrated a trend toward a survival advantage ($p = 0.09$). Clinically, there were no physical or behavioral differences noted between mice in the control group and those in the 3 treatment groups. Specifically, there were no statistically significant differences in constitutional signs, including animal weights in any of the treatment groups compared with control (data not shown), nor were there clinical signs of toxicity.

Discussion

Despite the plethora of targeted inhibitors identified and studied preclinically in GBM, appreciable improvements in patient survival have not followed.² One issue is the plasticity of GBM with its redundant signaling inputs²² and ability to bypass blockade of individual molecules by feedback loops.^{11,23} Paramount among these is the negative feedback loop by which inhibition of mTOR can trigger a negative feedback loop that results in PI3K/Akt activation. Therefore a combinatorial approach of PI3K inhibition, the common target for multiple RTKs, along with abrogation of mTOR to prevent feedback activation of Akt has been proposed and validated preclinically in multiple tumor types.^{24–29} XL765 is a novel, orally bioavailable PI3K/mTOR dual inhibitor that can be safely administered with no established maximally tolerated dose in phase I trials.²¹ In our study, we examined the effects

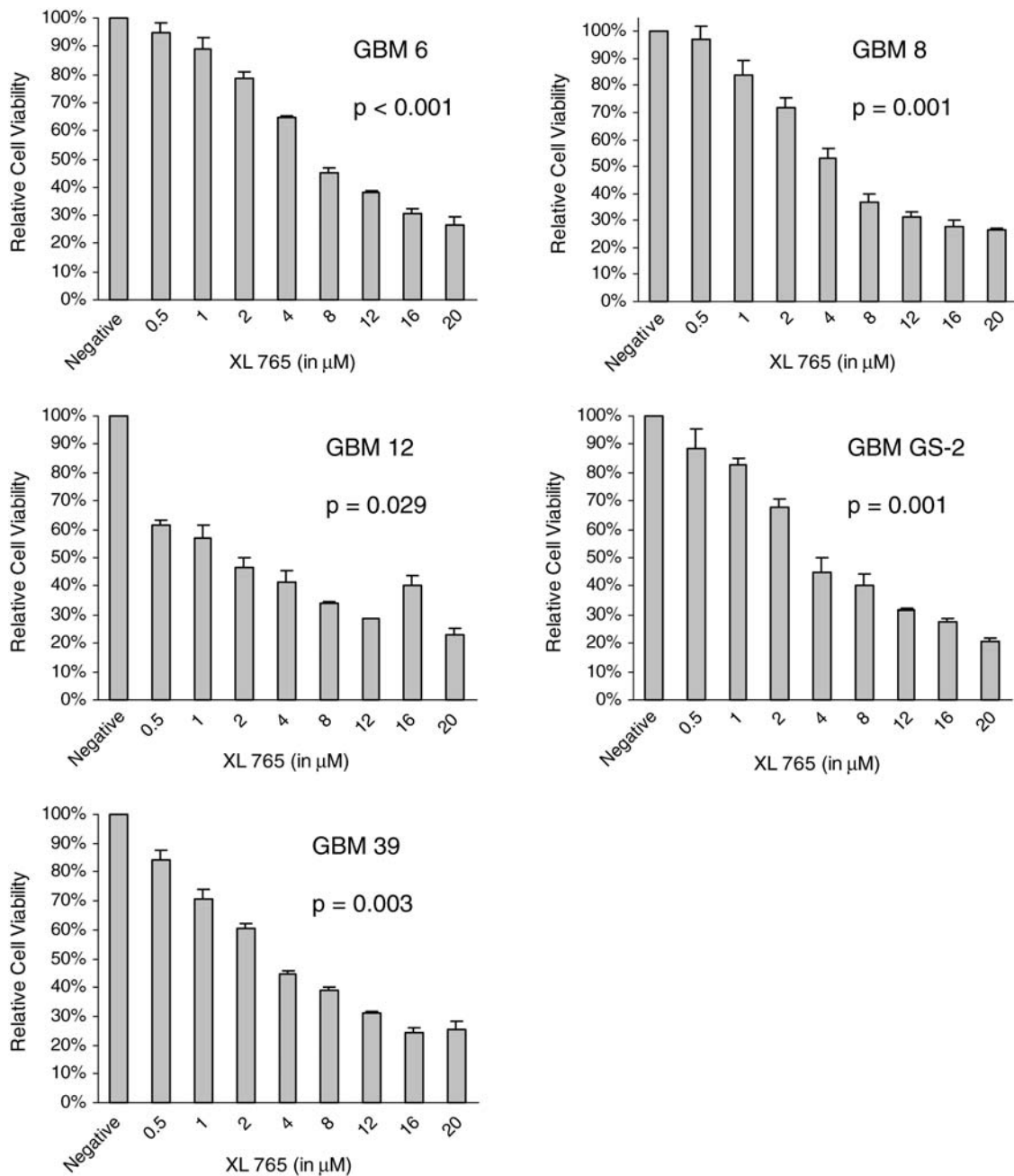


Fig. 2. Inhibition of PI3K and mTOR results in a concentration-dependent increase of cytotoxicity in human GBM xenografts cultured in vitro. Results are presented as the mean ± standard error of experiments done in triplicate. *p*-Values were generated using quadratic regression analysis.

of XL765 in a panel of genetically diverse GBM xenografts ± TMZ, the standard-of-care chemotherapeutic agent in postoperative GBM treatment.¹ To our knowledge, this is the first study to evaluate a PI3K/mTOR inhibitor in GBM using multiple, distinct, and characterized xenografts. Given the genetic heterogeneity of GBM, this is an important consideration to identify susceptible genotypes.

Regardless of genetic background, XL765 demonstrated specific inhibition of the PI3K/mTOR pathway (Fig. 1). All 5 xenografts showed highly robust inhibition

of pAkt (biomarker for PI3K) and pS6 (biomarker for mTOR), with >50% inhibition of phosphorylation within the 2- to 4-μM range. GBM 8 and GBM 12 (both of which are *EGFR wt* [amplified], *p16 null*, and *MGMT* hypermethylated) were relatively resistant to p4EBP1 modulation even at high concentrations of XL765. Expression of unrelated phosphoproteins pp38 and pJNK were also queried and showed no change after exposure to XL765 (data not shown). Similarly, there was no effect on non-phosphoproteins in the PI3K pathway.

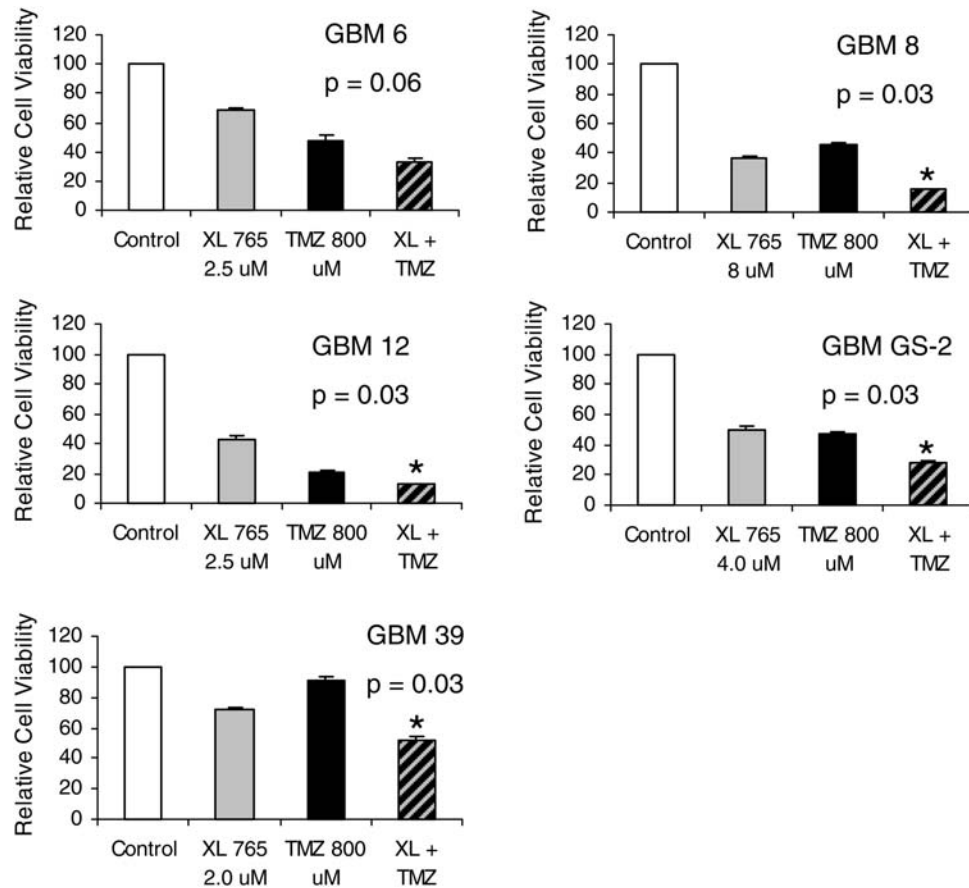


Fig. 3. Combinations of XL765 and TMZ result in additive cytotoxicity in 4 out of 5 GBM xenografts cultured in vitro. *p*-Values were generated using a Wilcoxon rank-sum test comparing XL765 + TMZ with the most effective single agent (XL765 or TMZ) in each GBM xenograft. * denotes $p < 0.05$.

Activation of the PI3K pathway has been shown to promote survival-related genes,³⁰ invasion of surrounding brain parenchyma,³¹ and recruitment of neural stem cells that are pro-invasion.³² Given this central role of PI3K in GBM, it is not surprising that XL765 resulted in in vitro cytotoxicity. Since TMZ is an important component of multimodality GBM treatment, we combined it with XL765 and showed statistically significant chemosensitization in 4 of 5 xenografts. To determine the mechanism of XL765-induced cytotoxicity, we performed several additional in vitro assays, including staining with Annexin V–fluorescein isothiocyanate to quantify apoptosis, propidium iodide staining to measure cell cycle effects, and clonogenic assays to evaluate reproductive cell death (data not shown). These experiments demonstrated equivocal results when XL765-treated cells were compared with controls. This was likely due to the fact that our xenografts were removed from animals and cultured in vitro for short periods of time and not used to establish primary cell cultures, which are more amenable to prolonged in vitro manipulation.

To evaluate the effects of XL765 ± TMZ in vivo, we injected mice intracranially with luciferase-expressing GBM 39 xenografts. We began treatments on day 21

after tumor injection, and our XL765 dosing schedule of 30 mg/kg was given twice per day (Monday–Friday, 6 h apart) for a period of 2 consecutive weeks without evidence of clinical toxicity or significant weight loss. A dosing schedule of 30 mg/kg twice per day was chosen over 100 mg/kg every other day due to preliminary evidence of increased toxicity with the latter dose (data not shown). XL765 showed benefits in growth reduction (~13 fold), survival, and in vivo reduction in pS6 expression by IHC compared with control, differences that were all statistically significant.

In GBMs that have *MGMT* hypermethylation, patients have improved outcomes particularly when TMZ is used.³³ Thus we were cognizant of the fact that our GBM 39 xenograft model (with *MGMT* hypermethylation) would likely be sensitive to TMZ. Despite this, combination of XL765 with TMZ still demonstrated a greater than 10-fold decrease in average tumor bioluminescence compared to TMZ alone. As seen in Figure 4B, levels of bioluminescence in the combination group were essentially the same as background, indicating very robust tumor regression. While mice in the combination group did have a longer median survival than mice receiving TMZ alone, this did not quite reach the level of statistical significance ($p = 0.09$).

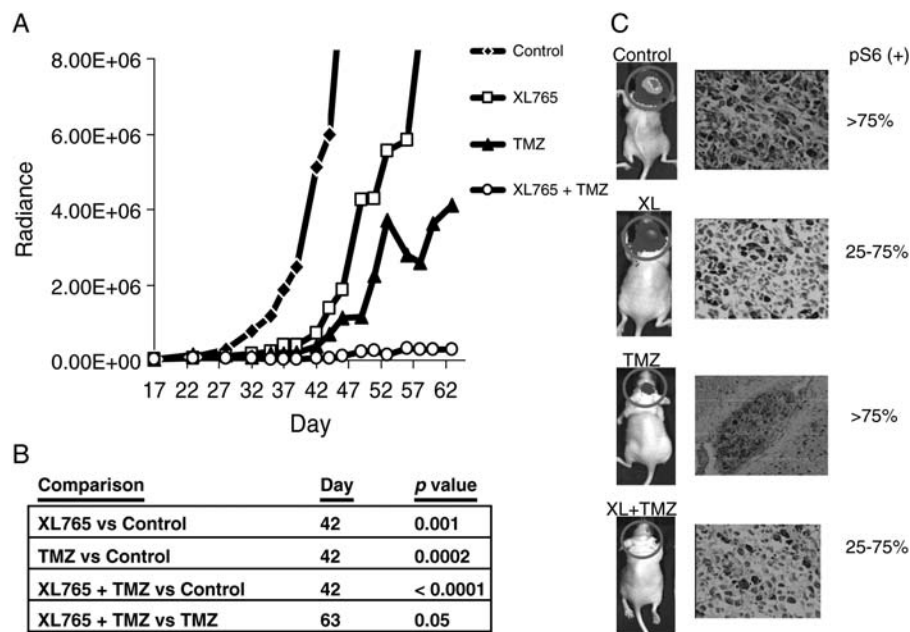


Fig. 4. Tumor burden as measured by bioluminescence is decreased after oral administration of XL765, TMZ, or XL765 + TMZ in nude mice bearing intracranial GBM 39 xenografts. Two weeks after xenograft injection, mice were randomized into 1 of 4 treatment groups. (A) Average bioluminescent values in each treatment group measured 3 times a week are shown until the last day on which all mice in the group were alive. (B) *p*-Values represent statistical comparisons between treatment groups using the Mann–Whitney test between independent samples; XL765 and TMZ groups were compared with control at day 46; XL765 + TMZ was compared with control at day 46 and with TMZ at day 63. (C) Representative bioluminescence images in each treatment group on day 46. Representative tumor sections stained for phosphorylated S6 are shown with samples treated with control or TMZ alone demonstrating >75% positive staining and tumors treated with XL765 alone or XL765 + TMZ demonstrating <75% positive staining.

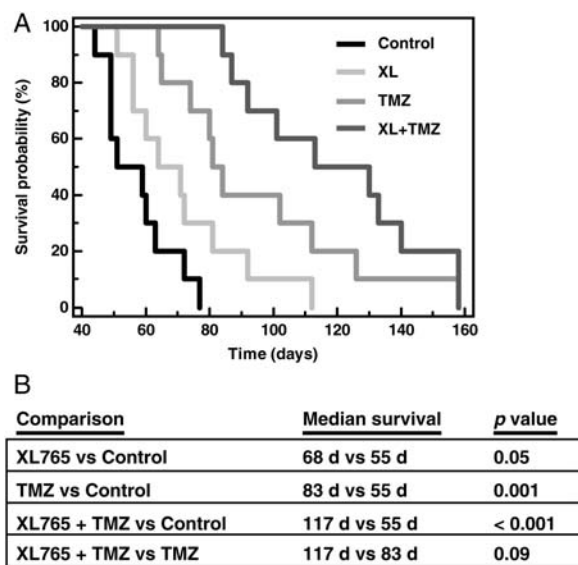


Fig. 5. Survival of mice increases after oral administration of XL765, TMZ, or XL765 + TMZ in nude mice bearing intracranial GBM 39 xenografts. (A) Kaplan–Meier survival curves. (B) Statistical comparisons using log-rank tests. XL765 and TMZ were compared with the control group, while XL765 + TMZ was compared with the control group and TMZ alone.

This is likely due to the *MGMT* status of GBM 39, which already contributes to baseline TMZ sensitivity.

In our study we have shown that a combined PI3K/mTOR inhibitor can be successfully utilized against GBM xenografts with a diverse genetic background. We hypothesize that XL765 would be most effective in patients with activating mutations in the PI3K pathway, specifically when *EGFR* is activated (through amplification of the wild-type form in GBMs 8, 12, and GS-2 or the VIII variant in GBMs 6 and 39) or *PTEN* is homozygously deleted (GBM 8 and GS-2). This hypothesis is based on two lines of reasoning: first, mTOR inhibitors are particularly effective against tumors with increased PI3K activity³⁹; second, erlotinib, a small-molecule inhibitor of epithelial growth factor receptor (*EGFR*), is most valuable in tumors with permissive genetic backgrounds that include activating *EGFR* mutations.^{37,38}

The utility of surrogate biomarkers in predicting responses to *EGFR* inhibitors in prospective clinical trials of GBMs is controversial. In a randomized trial by the European Organisation for Research and Treatment of Cancer comparing progression-free survival (PFS) using TMZ versus erlotinib in recurrent GBM, the activity of the latter could not be predicted by activating mutations in *EGFR* or expression of

pAkt or PTEN.⁴¹ Similar conclusions were reached in 2 other multi-institutional single-arm prospective clinical trials that failed to identify biomarkers that predict response to erlotinib in recurrent GBMs.^{42,43} In studies in which erlotinib has been used up-front with chemoradiation as part of primary GBM treatment, attempts to identify biomarkers have also resulted in contradictory findings: while a single-arm prospective study by the North Central Cancer Treatment Group⁴⁴ failed to find a relationship between biomarkers and tumor response, a similar study by UCSF⁴⁵ found an improvement in overall survival in a subset of patients with *MGMT* hypermethylation and intact *PTEN*. Though it may be tempting to conclude that failure of biomarkers to predict erlotinib activity in some of these trials implies a lack of benefit of EGFR inhibition in GBMs, there are several non-mutually exclusive alternate hypotheses: (1) use of a single EGFR inhibitor simply forces cancer cells to adapt by coactivating nontargeted tyrosine kinases,²² (2) there exists an excess toxicity of erlotinib,⁴⁶ (3) erlotinib exists in tumors in subtherapeutic concentration,⁴² and (4) insufficient patients were in surrogate biomarker groups to reach statistical significance.

The xenograft tested in our in vivo experiment had *EGFR vIII* amplification and wild-type *PTEN* and was indeed relatively sensitive to XL765. We would predict that a xenograft with *EGFR vIII* amplification and loss of *PTEN* would exhibit even greater sensitivity to XL765, and these experiments are ongoing. Furthermore, tumors without PI3K activation (granted,

a minority of GBMs) may exhibit less in vivo sensitivity to XL765, and we are testing this hypothesis. Genetic background has bearing on a second issue—that of sensitization of tumors to TMZ. In vitro, inhibition of the PI3K pathway sensitized xenografts to TMZ in nearly all cases; and in vivo, XL765 showed marked sensitization to TMZ in a xenograft with *MGMT* hypermethylated. Whether such dramatic sensitization to TMZ will be evident in GBMs without *MGMT* hypermethylated is a topic for further studies. Lastly, combining erlotinib with XL765 is intriguing from mechanistic and efficacy viewpoints, particularly given recent clinical data using the former in GBM.³⁴ Based in part on our preclinical data, a multi-institutional phase I trial studying the safety and tolerability of XL765 combined with TMZ is currently accruing.³⁵

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