Combination therapy of inhibitors of epidermal growth factor receptor/vascular endothelial growth factor receptor 2 (AEE788) and the mammalian target of rapamycin (RAD001) offers improved glioblastoma tumor growth inhibition

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Abstract
Malignant gliomas are highly lethal tumors that display striking genetic heterogeneity. Novel therapies that inhibit a single molecular target may slow tumor progression, but tumors are likely not dependent on a signal transduction pathway. Rather, malignant gliomas exhibit sustained mitogenesis and cell growth mediated in part through the effects of receptor tyrosine kinases and the mammalian target of rapamycin (mTOR). AEE788 is a novel orally active tyrosine kinase inhibitor that decreases the kinase activity associated with the epidermal growth factor receptor and, at higher concentrations, the vascular endothelial growth factor receptor 2 (kinase domain region). RAD001 (everolimus) is an orally available mTOR inhibitor structurally related to rapamycin. We hypothesized that combined inhibition of upstream epidermal growth factor receptor kinase and kinase domain region receptors with AEE788 and inhibition of the downstream mTOR pathway with RAD001 would result in increased efficacy against gliomas compared with single-agent therapy. In vitro experiments showed that the combination of AEE788 and RAD001 resulted in increased rates of cell cycle arrest and apoptosis and reduced proliferation more than either agent alone. Combined AEE788 and RAD001 given orally to athymic mice bearing established human malignant glioma tumor xenografts resulted in greater tumor growth inhibition and greater increases in median survival than monotherapy. These studies suggest that simultaneous inhibition of growth factor receptor and mTOR pathways offer increased benefit in glioma therapy.

Introduction
Glioblastomas remain essentially universally fatal despite maximal therapy with a median survival of only 10 to 12 months (1). Traditional treatments rely on cytotoxic therapies that achieve effects through damage of DNA or disruption of the mitotic machinery. Novel therapies under development inhibit the activities of specific molecular targets that contribute to the malignancy of cancers. Current clinical studies in neuro-oncology involve new therapeutic strategies that specifically target the unique molecular properties of gliomas. The epidermal growth factor receptor (EGFR) pathway is dysregulated in the majority of glioblastomas through overexpression, amplification, and/or mutation (2–5). EGFR plays important roles in the pathophysiology of glioblastomas, including the induction of cellular proliferation, motility, resistance to chemotherapy and radiation, and induction of neoangiogenesis. Expression of either wild-type or mutant EGFR may also contribute to tumor formation in genetic glioma models (6–10). EGFR function has been targeted through several techniques, prominently small molecule ATP-mimetic tyrosine kinase inhibitors (TKI). We reported recently the first completed trial of an EGFR TKI, gefitinib, in recurrent glioblastomas (11). Although a subset of patients experienced long-term control of tumor growth,
the majority of patients suffered progression of their tumors. Recent data suggest that tumors from lung cancer patients who display striking sensitivity to gefitinib contain mutations in the EGFR kinase region (12, 13). Mutational analysis of tumor specimens from patients with our trial on long-term tumor control (>24 weeks) did not yield any mutations (14), suggesting that kinase region mutations are not mechanisms by which EGFR inhibitor sensitivity can be predicted for glioblastoma patients. Thus, inhibition of EGFR alone will not be sufficient for the control of most glioblastomas.

The molecular mechanisms by which cancers develop resistance to EGFR inhibitors remain poorly understood. Whereas EGFR activates the phosphatidylinositol 3'-kinase (PI3K)/AKT signaling pathway, the tumor suppressor phosphatase and tensin homologue on chromosome 10 (PTEN, also known as MMAC1 or TEP1) inhibits the activation of AKT and increases sensitivity to apoptotic stimuli (15). Loss of PTEN activity has been associated with constitutively active AKT and cell transformation in many malignancies, including glioblastomas (16, 17). The mammalian target of rapamycin (mTOR) may lay downstream of AKT, and increased activity of mTOR may be detected in glioblastomas with constitutively active mutant EGFR (18) and in tumors with low PTEN activity. PTEN-deficient glioma cell lines display increased sensitivity to mTOR inhibition compared with those with wild-type PTEN (19). Recent studies of glioma cell lines resistant to EGFR TKIs have found increased activity of other signaling pathways, notably the insulin-like growth factor-I pathway (20, 21) as well as its downstream effectors, including PI3K and AKT/protein kinase B. Inhibiting EGFR with simultaneous blockade of either PI3K or AKT has shown benefit in inhibiting tumor cell proliferation (21–23). Loss of PTEN expression leads to constitutive activation of the PI3K/AKT signaling pathway and results in resistance to EGFR TKIs, which can be reversed with expression of PTEN or pharmacologic suppression of PI3K/AKT pathway activity (23, 24). Thus, the impact of EGFR inhibition may be augmented by therapies to which cells with PTEN loss may exhibit increased sensitivity. In particular, rapamycin analogues have shown preclinical and clinical efficacy in malignant gliomas with PTEN mutations (19, 25, 26).

Another mechanism by which resistance to growth factor receptor inhibitors may be induced includes the activation of parallel growth factor pathways. As EGFR may have significant impact on tumor growth through its proangiogenic effect, independent vascular endothelial growth factor receptor (VEGFR) activity may provide an important survival advantage with the withdrawal of EGFR effects (27). Thus, targeting EGFR and VEGFR in combination may offer additional therapeutic advantage. A recent study of pancreatic xenografts has shown that the combination of EGFR and VEGFR small-molecule TKIs offers increased benefit in tumor control (28). To create the broadest potential efficacy, we sought to define the potential efficacy of combining TKIs and mTOR antagonists in the treatment of malignant gliomas.

AEE788 (Novartis Pharma AG, Basel, Switzerland) is a 7H-pyrrolo[2,3-d]pyrimidine-class receptor TKI that potently inhibits the kinase activity associated with EGFR with additional inhibition of VEGFR-1 (Flt-1) and VEGFR-2 (kinase domain region/Flk-1) at higher concentrations. AEE788 exhibits antiproliferative activity against a broad spectrum of cancer cell lines (29). RAD001 (everolimus, Novartis Pharma) is a 40-O-(2-hydroxyethyl) derivative of rapamycin that exhibits improved aqueous solubility relative to the parent compound for oral administration (30). RAD001 has been well tolerated and efficacious in both preclinical and clinical transplant trials (31). RAD001 exhibits antiproliferative activity as well in preclinical cancer studies (32). Because a single tumor likely contains multiple aberrant cellular growth and survival pathways, blockade at one step in a given pathway is unlikely to provide lasting suppression of tumor growth. Combination chemotherapy was originally developed in the 1950s to combat the rapid development of resistance to single-agent therapy. We hypothesized that simultaneous inhibition of the upstream targets EGFR/VEGFR and the downstream target mTOR would result in greater inhibition of tumor growth than either agent alone. We have now shown that the combination of AEE788 and RAD001 offers combinatorial benefit in control of glioblastoma xenografts with suppression of downstream effectors.

Materials and Methods

Cells and Culture

The well-characterized human malignant glioma xenograft D54MG is the Duke University subline of A-172 (33). The transfected human glioblastoma cell lines U87MG.D2-7 and U87MG.wtEGRF, which express the exons 2 to 7 deletion mutant of EGFR and overexpress the wild-type EGFR, respectively, were derived from the parental U87MG line and have been described previously (7, 8). D54MG- and U87MG-derived human glioma cells were maintained by culturing in 10 cm tissue culture dishes in Zinc Option medium containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and glutamine (Life Technologies, Grand Island, NY) until ready for use.

Drugs

AEE788 and RAD001 were generously provided by Novartis Pharma. Stock solutions (10 mmol/L) were dissolved in DMSO (Sigma-Aldrich), stored at −80°C, and diluted in fresh medium immediately before use.

Antibodies and Western Blotting

For inhibitor studies, cells were pretreated with inhibitor for 1 hour, treated with either EGF or serum for 5 or 30 minutes, respectively, lysed in lysis buffer [62.5 mmol/L Tris-HCl, 2% w/v SDS, 10% glycerol, 40 mmol/L DTT], vortexed for 5 seconds, and centrifuged at 14,000 rpm for 5 minutes at 4°C. An equal amount of protein was run on polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and detected using an enhanced chemiluminescence system (Pierce Biotechnology, Rockford, IL). Phosphospecific EGFR
(Tyr\textsuperscript{1173}), phosphospecific ERK1/2 (Thr\textsuperscript{183}, Tyr\textsuperscript{185}), and total ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; ref. 18). Phosphospecific AKT (Ser\textsuperscript{473}), total AKT, total EGFR, phospho-Ser\textsuperscript{202}/Ser\textsuperscript{204} S6 ribosomal protein, and total S6 ribosomal protein antibodies were purchased from Cell Signaling Technology (Beverly, MA; ref. 18). α-Tubulin was purchased from Sigma-Aldrich. For immunohistochemical studies, an antiserum to EGFRvIII was used to determine the wild-type specific domain not present in EGFRvIII was used to determine wild-type levels.\textsuperscript{10} All antibodies were used according to the manufacturer’s instructions.

**Thymidine Incorporation Assays**

Cells ($n = 5,000$) were plated into each well of a 12-well tissue culture plate using Zinc Option medium containing 10% fetal bovine serum (Life Technologies) and glutamine (Life Technologies) for 24 hours. Next, the medium was changed to Zinc Option medium containing 0.1% bovine serum albumin (Life Technologies) for 18 hours to synchronize the cells. The medium was replaced with Zinc Option medium containing 10% fetal bovine serum (Life Technologies) and 2 mmol/L glutamine (Life Technologies) containing inhibitors for 72 hours. Cells were labeled for the last 3 hours with 4 Ci $[^3]$H]thymidine, fixed in 10% trichloroacetic acid, and lysed in 0.2 N NaOH. Thymidine incorporation into the DNA was measured with a scintillation counter. Each measurement was done in triplicate.

**Flow Cytometric and Annexin V Analysis**

Cells ($n = 200,000$) were plated into a 10 cm tissue culture dish. The cells were serum starved overnight and treated with inhibitors in Zinc Option medium containing 10% fetal bovine serum (Life Technologies) and 2 mmol/L glutamine (Life Technologies) for 72 hours. The cells were then removed from the plate, fixed in ethanol, and stained with propidium iodide. Analysis was done on FACScan and gated to exclude cellular debris; collecting 10,000 events, calculations were done using with BD software. For Annexin V analysis, 100,000 cells were plated in six-well plates, serum starved overnight with inhibitors, collected for analysis, washed twice with Dulbecco’s PBS, and stained with propidium iodide and Annexin V (BD PharMingen, San Diego, CA) per manufacturer’s instructions.

**Statistical Analysis**

Data in all in vitro studies were done at least in triplicate and compared by using the nonparametric Wilcoxon rank-sum test (34).

**Intracranial Xenograft Studies**

Athymic male BALB/c nu/nu mice were maintained in the Duke University Cancer Center Isolation Facility according to institutional policy. Subcutaneous xenografts passed in athymic mice were excised, minced, and dissociated with 0.5% collagenase at room temperature in a trypsinization flask for 40 minutes. For intracranial model studies, viable cells were separated on a Ficoll density gradient, washed twice with Dulbecco’s PBS, resuspended in 2.5% methylcellulose at a concentration of $1 \times 10^7$ cells/mL, and injected into the implanted guide cannula through the 33-gauge infusion cannula in a volume of 5 mL by using a 500 L gas-tight syringe and injector (Hamilton Co., Reno, NV).

**Subcutaneous Xenograft Transplantation**

Subcutaneous tumor transplantation was done into the right flank of the animals with an inoculation volume of 50 μL using a brei prepared from xenografts (35).

**Tumor Measurements**

Tumors were measured twice weekly with hand-held Vernier calipers (Scientific Products, McGraw, IL). Tumor volume was calculated according to the following formula: $V = \left[\frac{\text{width}}{2}\right]^2 \times \left[\frac{\text{length}}{2}\right]$.

**Drug Toxicity**

Mice were weighed twice weekly to assess weight loss and were checked daily for survival.

**Xenograft Therapy**

For s.c. tumor studies, groups of 10 mice randomly selected based on tumor volume were treated when the median tumor volume was in the range of 100 to 300 mm\textsuperscript{3} and were compared with control animals receiving drug vehicle. For intracranial tumor studies, groups of 10 mice were randomized 3 days after intracranial tumor implantation. AEE788 was given at a dose of 100 mg/kg thrice a week per gavage. RAD001 was given at a dose of 5 mg/kg thrice a week per gavage. When AEE788 and RAD001 were used in combination, AEE788 was given 1 hour before RAD001.

**Tumor Response Assessment**

The response of the s.c. xenografts was assessed by delay in tumor growth and by tumor regression. Growth delay (expressed as $T - C$) is defined as the difference in days between the median time required for tumors in treated ($T$) and control ($C$) animals to reach a volume five times greater than that measured at the start of treatment. Partial tumor regression is defined as a decrease in tumor volume over at least two successive measurements. The response of the intracranial xenograft studies was assessed as the difference in the median duration of survival. Statistical analyses were done using a personalized SAS statistical analysis program, the Wilcoxon rank-order test for growth delay, and Fisher exact test for tumor regression as described previously (36). Survival estimates and median survivals were determined by using the method of Kaplan and Meier.

**Immunohistochemistry**

Athymic nude mice with established s.c. D54MG xenografts were treated daily with vehicle control (DMSO), AEE788 (200 mg/kg per gavage), RAD001 (5 mg/kg per gavage), or the combination of AEE788 and RAD001 for 5 days. As the resting EGFR phosphorylation in tumor cells is low, mice were injected i.p. with 100 μg human recombinant EGF (Sigma-Aldrich) 1 hour after the final

\textsuperscript{10} C. Wikstrand, unpublished data.
treatment and were sacrificed after 5 minutes. Tumors were harvested, and portions were fixed in formalin for 24 hours, transferred to 70% ethanol, and sectioned for staining with H&E or immunohistologic analysis with antibodies against Ki-67, phosphaminoacid-EGFR or total-EGFR, AKT, ERK1/2, S6 ribosomal protein, and mTOR.

Formalin-fixed, paraffin-embedded sections were used for analysis with antibodies directed against EGFR, pEGFR, ERK1/2, pAKT, pmTOR, S6, and pS6. Our standard methodology for formalin-fixed, paraffin-embedded sections (36) was used with the following exceptions, incorporating the manufacturer’s suggestions. For antibodies to pmTOR (3.2 μg/mL), ERK1/2 (0.5 μg/mL), pERK1/2 (1 μg/mL), AKT (5 μg/mL), and pAKT (1.5 μg/mL), antigen retrieval was done using microwave treatment with 10 mmol/L citrate buffer (pH 6.0, 2 minutes/8 minutes); for antibodies to EGFRwt (5 μg/mL), pEGFR (1.2 μg/mL), S6 (2 μg/mL), and pS6 (0.2 μg/mL), antigen retrieval was done using microwave treatment with 1 mmol/L EDTA (pH 8.0, 2 minutes/13 minutes) following deparaffinization and rehydration. Purified normal rabbit IgG was titrated over the concentrations required for each antigen retrieval procedure. Slides were then blocked for endogenous peroxide activity and with 20% normal goat serum for 1 hour at room temperature; the remainder of the assay has been described previously (37, 38).

Immunoreactivity was independently graded by two investigators. An intensity score of the immunoreactivity was graded on a range of absent (0), mild (1), moderate (2), and strong (3). Distribution of the immunoreactivity was also noted as focal, multifocal, and diffuse as described previously (38). In most cases, strong immunoreactivity of the antigen in question could be identified in endothelial cells of the tumors both verifying the reactivity of the antibody and establishing an internal control. Negative controls consisted of tissues treated with the entire antibody protocol, except for a monoclonal antibody to an irrelevant antigen substituted for the positive antibody at the appropriate dilution.

**Ki-67 Quantification**

For every tumor evaluated, 20 high-powered fields with viable areas of tumor were selected by a blinded observer. Positive nuclei were quantified using Image J.11 Data were compared by using the nonparametric Wilcoxon rank-sum test (34).

**Results**

**AEE788 Inhibits EGFR Phosphorylation and Proliferation of Human Glioma Cell Lines**

To examine the contributions of EGFR and mTOR in the proliferation of malignant gliomas, we used the well-characterized human malignant glioma D54MG cell line for these experiments. D54MG cultures express ~9 × 10⁴ wild-type EGFR receptors/cell but do not express kinase domain

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Figure 1. AEE788 inhibits EGFR and proliferation of human glioma cell lines. **A**, D54MG cultures were pretreated for 1 h with increasing concentrations of AEE788 followed by a 5-min incubation with 10 pmol/L EGF. Whole cell lysates were collected, resolved by SDS-PAGE, and immunoblotted with phosphospecific antibodies. Membranes were stripped and reprobed with antibodies to measure total levels of each protein. Equal protein loading was confirmed by tubulin immunoblotting. **B**, D54MG cells were plated in 12-well plates at a cellular density of 5,000 cells per well. Cells were treated with AEE788 concentrations in triplicate wells for 72 h in serum-fed conditions. Cells were labeled with tritiated thymidine for the last 3 h of treatment. *, P < 0.05, compared with control. **C** and **D**, D54MG cultures were treated with either DMSO control (0.1%) or AEE788 (10 μmol/L) for 72 h, harvested, permeabilized with ethanol, and labeled with propidium iodide. Flow cytometric analysis of cell cycle fractions was done (C, sub-G1; D, G2). *, P < 0.05, compared with control by nonparametric Wilcoxon rank-sum test.
We began these experiments by measuring the effect of AEE788 mono-therapy on the level of activated phosphorylation of the target EGFR in D54MG cultures. As shown in Fig. 1A, increasing doses of AEE788 resulted in significant reduction in the levels of phosphorylated EGFR, whereas the amount of total EGFR did not change significantly with drug treatment. EGFR promotes cellular proliferation and resistance to apoptotic stimuli through intracellular mediators, including ERK and AKT. AKT phosphorylation levels induced in response to EGF treatment were decreased by treatment with AEE788 (Fig. 1A). ERK phosphorylation was less clearly induced by EGF treatment and only decreased by AEE788 at the highest doses (Fig. 1A), suggesting that ERK activation is not EGFR dependent in this cell line. To determine the impact of AEE788 on cellular proliferation, DNA synthesis of D54MG cultures treated with AEE788 was measured by thymidine incorporation (Fig. 1B). AEE788 concentrations of 8 to 9 μmol/L were required to inhibit DNA synthesis by 50%. Higher concentrations of AEE788 (15–20 μmol/L) induced >95% decrease in thymidine incorporation (data not shown). To determine the cellular effect responsible for the decreased DNA synthesis with AEE788 treatment, D54MG cultures were labeled with propidium iodide and analyzed by flow cytometry. AEE788 treatment (10 μmol/L) resulted in at least 2-fold increase in the proportion of cells in the sub-G0 fraction and Annexin V/propidium iodide–positive cells (Figs. 1C and 2), which is consistent with drug-induced apoptosis, and increased the proportion of cells in the G2 phase (Fig. 1D), consistent with G2 arrest. High concentrations of AEE788 (15–20 μmol/L) induced necrotic cell death (Fig. 2; data not shown). Thus, AEE788 shows activity against D54MG cultures with both anti-proliferative and proapoptotic effects. The excess concentration of AEE788 required to suppress D54MG proliferation above that required to suppress EGFR phosphorylation suggests that these cells are partially resistant to EGFR blockade in line with prior studies (21).

RAD001 Inhibits Activation of mTOR Targets and Inhibits Proliferation

D54MG glioma cells express wild-type TP53 and a nonfunctional form of the tumor suppressor PTEN with deletion of exons 3 to 9 (ref. 39; data not shown). Due to the loss of this negative regulator of the PI3K pathway, the mTOR signaling pathway and its downstream elements are likely more active in D54MG cultures than in normal human tissue. As mutations of PTEN are associated with increased sensitivity to rapamycin analogues (19), we expected that D54MG cultures would be sensitive to RAD001. Indeed, we found that the phosphorylation of an important effector of mTOR, S6 ribosomal protein, was decreased by nanomolar concentrations of RAD001 (Fig. 3A). As expected, there was a more modest impact on EGFR and AKT phosphorylation (Fig. 3A). In parallel, RAD001 inhibited DNA synthesis significantly at nanomolar concentrations with 50% inhibition at 2 to 3 nmol/L (Fig. 3B). The concentrations required to achieve specific levels of inhibition were 2- to 3-fold higher for RAD001 than for rapamycin (data not shown), but both RAD001 and rapamycin maximally inhibited DNA synthesis at 75% to 80% of control levels even at micromolar concentrations (Fig. 3B; data not shown). Unlike AEE788, RAD001 treatment induced an increase in G1 cell cycle fraction without appreciable cell death on either flow cytometry (Figs. 2 and 3C) or cellular morphology. The fact that RAD001 and AEE788 both inhibited cellular proliferation through apparently different mechanisms provided further rationale for combining these two agents.

Combination of AEE788 and RAD001 Inhibits Downstream Targets

Concentrations of AEE788 and RAD001 capable of partially reducing activation of downstream signal transduction molecules were selected for use in combination.

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12 C. Wikstrand et al., unpublished data.
As expected, AEE788 treatment potently decreased EGFR phosphorylation induced in response to EGF treatment in either the absence or the presence of RAD001 (Fig. 4). Not surprisingly, RAD001 treatment had marginal impact on EGFR, ERK, or AKT phosphorylation. AEE788 decreased AKT phosphorylation with a lesser impact on ERK phosphorylation with EGF treatment independent of RAD001 treatment (Fig. 4). Serum treatment had an increased AKT phosphorylation but minimal effects on EGFR and ERK phosphorylation (Fig. 4), suggesting that serum activates AKT predominantly by growth factor pathways other than EGFR in D54MG cultures. AEE788 had a modest impact on the phosphorylation of S6 ribosomal protein, whereas RAD001 potently decreased phosphorylation in response to serum treatment (Fig. 4). These results suggest that the combination of AEE788 and RAD001 does not interfere with the ability of either agent to inhibit downstream effectors of its primary target. This is an important issue, as some targeted therapies require the cell to enter specific cell cycle points to induce therapeutic effects.

### Impact of Wild-type or Mutant EGFR Expression on Effects of AEE788 and RAD001

We further sought to determine the impact of wild-type and constitutively active mutant forms of EGFR on the response to these therapies, as some cell types have shown resistance to EGFR TKIs with the expression of mutant EGFR (40). For these studies, we used a set of well-characterized human malignant glioma cell lines that were genetically modified to express either form of EGFR. Parental U87MG cultures have wild-type TP53, are PTEN mutant, and have moderate expression of wild-type EGFR (4/C2104 receptors per cell) and no EGFRvIII expression. 13

As expected, AEE788 treatment potently decreased EGFR phosphorylation induced in response to EGF treatment in either the absence or the presence of RAD001 (Fig. 4). Not surprisingly, RAD001 treatment had marginal impact on EGFR, ERK, or AKT phosphorylation. AEE788 decreased AKT phosphorylation with a lesser impact on ERK phosphorylation with EGF treatment independent of RAD001 treatment (Fig. 4). Serum treatment had an increased AKT phosphorylation but minimal effects on EGFR and ERK phosphorylation (Fig. 4), suggesting that serum activates AKT predominantly by growth factor pathways other than EGFR in D54MG cultures. AEE788 had a modest impact on the phosphorylation of S6 ribosomal protein, whereas RAD001 potently decreased phosphorylation in response to serum treatment (Fig. 4). These results suggest that the combination of AEE788 and RAD001 does not interfere with the ability of either agent to inhibit downstream effectors of its primary target. This is an important issue, as some targeted therapies require the cell to enter specific cell cycle points to induce therapeutic effects.
The induction of phosphorylation of EGFR in response to EGF in the parental U87MG cells was very low, so the detection of the impact of AEE788 was not reliably assessed. We have therefore used genetically modified cultures to more reliably probe the contributions of both wild-type and mutant EGFR to AEE788 response. EGF treatment induces an increase in EGFR phosphorylation of U87MG.wtEGFR, whereas U87MG.D2-7 expressed constitutively active mutant EGFR with a slight response of wild-type EGFR (Fig. 5; data not shown). Although AEE788 treatment decreased EGFR phosphorylation of both U87MG.wtEGFR and U87MG.D2-7 cultures, U87MG.wtEGFR cultures were more sensitive to AEE788 than U87MG.D2-7 in terms of AKT phosphorylation changes, whereas U87MG.D2-7 was more sensitive to suppression of ERK phosphorylation by AEE788 than U87MG.wtEGFR (Fig. 5; data not shown). This is consistent with the observation that expression of wild-type EGFR in glioma specimens is associated with activation of ERK, whereas EGFRvIII is more closely linked to PI3K/AKT pathway activation (18). The biological consequences of the differences between U87MG.wtEGFR and U87MG.D2-7 did not affect the effects of AEE788 on cellular proliferation, as thymidine incorporation assays of U87MG.wtEGFR and U87MG.D2-7 showed essentially identical inhibition at equal concentrations (data not shown). In contrast, RAD001 treatment of both U87MG.wtEGFR and U87MG.D2-7 cultures decreased S6 ribosomal protein phosphorylation at low nanomolar concentrations with greater concentrations of RAD001 to suppress U87MG.D2-7 (Fig. 5).

Additionally, RAD001 treatment displayed an increase in phosphorylated AKT with serum stimulation, suggesting a compensatory up-regulation of targets upstream from mTOR. This effect of RAD001 has been reported previously (41). Combinations of low-dose AEE788 and RAD001 treatments affected AKT, ERK, and S6 ribosomal protein phosphorylation similar to each as monotherapy (Fig. 5). U87MG.wtEGFR or U87MG.D2-7 showed similar combinatory benefit in inhibition of DNA synthesis with the combination of AEE788 and RAD001 (data not shown). Thus, the expression of EGFRvIII does not seem to preclude functional sensitivity to either AEE788 or AEE788 in combination with RAD001. The proliferation of all U87MG cell lines does not depend completely on solely ERK or AKT pathways, as decreased phosphorylation of ERK and AKT is only moderately linked to changes in proliferation. This suggests that U87MG cells have redundant proliferative pathways incompletely targeted by AEE788.

**Figure 5.** Effects of wild-type and mutant forms of EGFR on response to AEE788 and RAD001. U87MG.wtEGFR and U87MG.D2-7 cultures were pre-treated with AEE788 (1 μmol/L) ± RAD001 (1 nmol/L) for 1 h followed by 5-min incubation with EGF (10 pmol/L) or 30-min incubation with 10% fetal bovine serum. Whole cell lysates were collected, resolved by SDS-PAGE, and immunoblotted with phosphospecific antibodies. Membranes were stripped and reprobed with antibodies to measure total levels of each protein. Equal protein loading was confirmed by α-tubulin immunoblotting.

**AEE788 and RAD001 Block Cellular Proliferation in Cell Culture**

To examine the combinatorial benefit of AEE788, we did a 2 × 2 thymidine incorporation experiment using D54MG cultures (Fig. 6A). As above, AEE788 and RAD001 monotherapies decrease thymidine incorporation, indicating decreased DNA synthesis and decreased cellular proliferation. The addition of each tested concentration of AEE788 induced a decrease in thymidine incorporation for every concentration of RAD001, and RAD001 concentrations of either 1 or 10 nmol/L improved the effects of AEE788 at 7.5 and 10 μmol/L but not at 12.5 μmol/L at which concentration cellular proliferation is already inhibited at >95%. Flow cytometric analysis of serum-fed D54MG cultures treated with AEE788 alone, RAD001 alone, or the combination showed a modest increase in
G1 cell cycle fraction with either RAD001 alone or in combination with AEE788 (Fig. 6B). AEE788 treatment was associated with an increase of apoptosis either alone or in combination with RAD001 (Figs. 2 and 6C). A combinatory impact of AEE788 and RAD001 treatment was seen in the suppression of cellular S-phase fraction (Fig. 6D). In summary, the combination of AEE788 and RAD001 offers increased inhibition of tumor cell proliferation in vitro.

AEE788 and RAD001 Inhibit Tumor Growth

Orally dosed AEE788, RAD001, and the combination were well tolerated by tumor-bearing athymic mice without significant weight loss but less weight gain than other treatment arms (data not shown). Rare toxic deaths were seen with 5 mg/kg RAD001 treatment given thrice a week (1 of 10 mice in each of two of three trials). No toxic deaths were seen in mice treated with the combination of AEE788 and RAD001. In a heterotopic xenograft model of established s.c. tumors, AEE788 (100 mg/kg thrice a week) and RAD001 (5 mg/kg thrice a week) monotherapies each modestly slowed D54MG xenograft growth in three trials (representative results; Fig. 7). However, the combination was significantly more effective than either agent alone in inhibiting D54MG tumor growth. As measured by time to reach five times initial tumor volume, AEE788 delayed tumor growth by 5.2 days ($P = 0.022$ relative to control), RAD001 by 7.3 days ($P = 0.014$), and the combination by 31.7 days ($P = 0.001$). As defined by a decreased tumor size on two sequential volumetric measurements, partial tumor regressions were only seen with combination therapy (5 of 10 treated animals in two studies and 4 of 10 in a third study). No tumors underwent complete regression, and all tumors eventually regrew. Survival of mice with implanted intracranial tumors is used as a clinical surrogate for tumor growth. In an intracranial D54MG xenograft study, monotherapy with AEE788 and RAD001 led to moderate increases in median animal life span in replicate studies (representative trial shown in Fig. 8). Once again, the combination yielded further increases in life span with median life spans of 20 days for control mice, 26.5 days for AEE788 treated animals, 25 days for RAD001 treated animals, and 29 days for combination treated animals ($P = 0.032$ against control, $P = 0.095$ against AEE788 monotherapy, and $P = 0.045$ against RAD001 monotherapy).

Immunohistochemical Analysis of Tumors Treated with Inhibitors

We examined the consequences of AEE788 and RAD001 treatments on the histologic appearance of established s.c. D54MG xenografts treated with a course of orally given therapies. Control tumors displayed hypercellular areas with a high mitotic rate (measured by percentage of cells expressing Ki-67). RAD001 monotherapy did not significantly affect proliferation, but AEE788 monotherapy and in combination with RAD001 decreased cellular proliferation as measured by Ki-67 staining (Fig. 9). To evaluate the selective inhibition of the targets of these therapies, we did immunohistochemical analysis of phosphorylated EGFR and mTOR and their mediators. Immunohistochemical reactivity for EGFR and its downstream activated (phosphorylated) messenger system, including ERK, AKT, and S6 ribosomal protein, reveal moderate to strong reactivity in the control D54MG xenografted tissue, indicating that the system is both activated and that immunohistochemical detection of these epitopes is a successful technique. In all tissues tested (controls and treated), reactivity against the total (phosphorylation insensitive) forms of the antigens were either stronger or equal in reactivity to the phosphorylated (activated) forms of the respective antigens. With the exception of mTOR,
for which nonphosphorylated target was not examined, in the treated tissues, the reactivity for the total antigens often exhibited markedly stronger immunoreactivity than the phosphorylated forms, indicating that the phosphorylated forms were effectively diminished in quantity versus nontreated controls (Fig. 10).

The results indicate that AEE788 effectively abolished reactivity for pEGFR and pERK, diminished reactivity for pmTOR, and had little or no effect on the detected levels of pAKT or pS6. These results are notably different from cell culture results, as ERK in D54MG xenografts exhibited dependence on receptor tyrosine kinase activity, whereas AKT was quite resistant to inhibitor therapy; this is exactly opposite to the cell culture results (Fig. 1). Treatment with RAD001 had little or no effect on the detected levels of pEGFR or pERK and diminished pAKT immunoreactivity from a strong diffuse pattern to a strong multifocal pattern. pmTOR staining was moderately to markedly diminished compared with untreated controls. There was no detectable expression of S6 or pS6 in RAD001-treated xenografts. The combination of AEE788 and RAD001 resulted in losses of detectable levels of pEGFR, pS6 staining, and moderately diminished reactivity for pmTOR. There was no effect on pERK or pAKT reactivity. Of note, the histologic grading

Figure 7. Combination of AEE788 and RAD001 decreases in vivo tumor growth. D54MG xenografts were grown in the flanks of athymic nude mice until a tumor volume of 100 to 300 mm$^3$ was reached. Animals were treated per gavage with AEE788 (100 mg/kg thrice a wk) ± RAD001 (5 mg/kg thrice a wk). A, mice were weighed twice weekly. A modest but statistically nonsignificant difference in mouse weights was seen. B, tumor volume was also measured with calipers twice a wk. Tumor volume was calculated by the following calculation: volume = (smallest diameter)$^2$ (largest diameter) / 2.

Figure 8. Combination of AEE788 and RAD001 increases survival of animals with intracranial tumors. D54MG xenografts implanted into nude athymic mice until a tumor volume of 100 to 300 mm$^3$ was reached. Treatment was gavaged with AEE788 (100 mg/kg thrice a wk) ± RAD001 (5 mg/kg thrice a wk). Control, AEE788, RAD001, and combination therapy. D54MG xenografts were treated with vehicle control, AEE788 (100 mg/kg thrice a wk) ± RAD001 (5 mg/kg thrice a wk), or the combination per gavage for 3 d. One hour after the final dose, mice were treated with EGF (100 ng i.p.). After 5-min incubation, tumors were harvested, fixed in formalin, and stained for Ki-67 as a marker of proliferation. Areas of viable tumor were examined for positive cells (brown). A, control; B, AEE788 monotherapy; C, RAD001 monotherapy; D, AEE788 and RAD001 combination therapy; E, quantification of Ki-67-positive nuclei per high-powered fields (hpf) was measured in 20 fields per tumor sample. *, $P = 0.011$ versus control. **, $P = 0.0048$ versus control, $0.0076$ versus AEE788 alone, and $<0.001$ versus RAD001 alone.
system employed, which determined a score by multiplying an overall cellular distribution index by a staining intensity index, revealed that combination chemotherapy resulted in a decreased cellular distribution of phosphorylated mTOR with a higher intensity in the few immunoreactive (possibly drug insensitive) cells (Fig. 10).

Discussion
We now show that the combination of two molecular targeted therapies—the EGFR/VEGFR2 inhibitor AEE788 and the mTOR inhibitor RAD001—offers a therapeutic advantage over either agent as monotherapy for glioma therapy. As EGFR and mTOR have each been proposed as therapeutic targets in gliomas, both AEE788 and RAD001 are each in clinical trial for glioma patients. Our results suggest that a combination of AEE788 and RAD001 may offer additional benefit. Indeed, a clinical trial using this approach is now accruing at our institution.

Combination cancer therapies are frequently designed to target tumor growth with nonoverlapping mechanisms to better overcome resistance. As expected, AEE788 and RAD001 blocked the activation of different oncogenic pathways in in vitro studies. Additionally, AEE788 and RAD001 affected cellular proliferation and apoptosis in different ways as well. AEE788 blocked EGFR phosphorylation in response to EGF stimulation and, at higher concentrations, the phosphorylation of the downstream effectors AKT and ERK. The requirement of AEE788 concentrations more than that required to suppress EGFR phosphorylation to inhibit cell proliferation suggests that AKT and ERK activation are not exclusively dependent on EGFR activity. In fact, AKT and ERK are phosphorylated at a basal state in the absence of EGF treatment (Fig. 1A). These results are in line with previous studies, demonstrating that most glioma cell lines are resistant to the EGFR TKI, gefitinib, and growth inhibition with gefitinib treatment occurs at concentrations well above those required to block EGFR activation (21). Malignant gliomas cultured in normal cell culture conditions lose both cellular expression of EGFR and mitogenic dependence on EGFR, whereas gliomas maintained in vivo as xenografts maintain EGFR expression and dependence (42). Even with the forced expression of EGFR or EGFRvIII, we showed that U87MG cell lines required excess concentrations of the EGFR TKI AEE788 to suppress cellular proliferation beyond the concentrations required to block EGFR activation. Therefore, caution must be exercised in the interpretation of cell

![Figure 10. Immunohistochemical analysis of tumor xenografts treated with AEE788 and RAD001. Nude athymic mice with established D54MG xenografts were treated with vehicle control, AEE788 (200 mg/kg/d), RAD001 (5 mg/kg/d), or the combination per gavage for 5 d. One hour after the final dose, mice were treated with EGF (100 µg i.p.). After 5-min incubation, tumors were harvested and formalin fixed. Tumor sections were stained with antibodies to both unphosphorylated and phosphorylated targets as described in Materials and Methods. Representative microscopic fields of each immunohistochemical reaction with the overall semiquantitative grading scale (×400).](attachment:figure_10.png)
culture studies used to determine the efficacy of EGFR targeting agents against malignant gliomas. We therefore performed xenograft studies that also show activity of AEE788 against malignant gliomas. AEE788 blocks increase tumor cell number to a greater degree than RAD001 in both cell culture and animal studies. AEE788 induces apoptosis independent of RAD001 treatment, suggesting that these agents can be combined effectively, whereas other studies using combination of molecular targeted agents display less than additive benefit due to activation of AKT with mTOR blockade by RAD001. The effects of AEE788 on progression through G1 cell cycle phase and RAD001 on progression through the G2 phase are modest but are cumulative enough to significantly inhibit cellular proliferation. As AEE788 and RAD001 display differential effects on the cell cycle, the combination of these agents do not seem to disrupt the effects of one another.

Although xenograft studies are not fully predictive of the therapeutic efficacy of cancer therapies in clinical trials, xenograft studies may offer additional information over cell culture studies. We have found that the phosphorylation of AKT and ERK in response to AEE788 treatments differ between in vitro and in vivo assays. Whereas AKT phosphorylation was inhibited at modest concentrations of AEE788 in cell culture, little impact of AEE788 on AKT activation was detected in xenografted tumors. On the other hand, AEE788 only marginally inhibited ERK phosphorylation even at high concentrations in cell culture conditions, whereas AEE788 treatment of xenografts readily lowered expression of phosphorylated ERK. Further, xenografts treated with the combination of AEE788 and RAD001 displayed focal areas of intense ERK and mTOR phosphorylation, suggesting that tumors that survive combination therapy may activate these pathways focally. These findings imply that tumors may activate feedback or compensatory pathways to promote tumor growth and survival. In fact, the impact of the therapy on its putative targets may be underestimated in our studies, as the immunohistochemical targets that we measured only reflect the effects of the drugs on the remaining viable cells. Cells exquisitely sensitive to the drug(s) may have died, effectively diminishing the sensitivity of our assay. These results further show the need to perform both cell culture and xenograft studies in the preclinical development of targeted therapies.

Our results suggest that combination of targeted therapies currently under clinical development may offer therapeutic benefit in glioma therapy. Joint inhibition of upstream receptor tyrosine kinases and intracellular effectors may act to increase sensitivity of tumor cells to the effects of each agent. Besides EGFR, several other growth factor receptors are active in gliomas, including VEGFRs and platelet-derived growth factor receptor, suggesting that small-molecule inhibitors of these receptors may offer combinatorial benefit with mTOR antagonists. The direct implications of these findings include the theoretical basis for novel clinical trials combining mTOR antagonists with small-molecule inhibitors of growth factor receptors. Of larger consequence, the combination of small molecules with limited clinical efficacy may offer significant benefit. Novel signal transduction inhibitors that fail initial clinical development, as monotherapies may represent useful therapies in combination with other agents. It is conceivable that future oncology treatments will involve characterization of activity of signaling intermediates and customized inhibitor cocktails perhaps in combination with cytotoxic therapies.

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References

Inhibitors of EGFR/VEGFR2 and mTOR in Glioma Therapy


Combination therapy of inhibitors of epidermal growth factor receptor/vascular endothelial growth factor receptor 2 (AEE788) and the mammalian target of rapamycin (RAD001) offers improved glioblastoma tumor growth inhibition

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