Dual mTORC1/2 Blockade Inhibits Glioblastoma Brain Tumor Initiating Cells In Vitro and In Vivo and Synergizes with Temozolomide to Increase Orthotopic Xenograft Survival

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Abstract

Purpose: The EGFR and PI3K/mTORC1/2 pathways are frequently altered in glioblastoma (GBM), but pharmacologic targeting of EGFR and PI3K signaling has failed to demonstrate efficacy in clinical trials. Lack of relevant models has rendered it difficult to assess whether targeting these pathways might be effective in molecularly defined subgroups of GBMs. Here, human brain tumor–initiating cell (BTIC) lines with different combinations of endogenous EGFR wild-type, EGFRvIII, and PTEN mutations were used to investigate response to the EGFR inhibitor gefitinib, mTORC1 inhibitor rapamycin, and dual mTORC1/2 inhibitor AZD8055 alone and in combination with temozolomide (TMZ).

Experimental Design: In vitro growth inhibition and cell death induced by gefitinib, rapamycin, AZD8055, and TMZ or combinations in human BTICs were assessed by alamarBlue, neurosphere, and Western blotting assays. The in vivo efficacy of AZD8055 was assessed in subcutaneous and intracranial BTIC xenografts. Kaplan–Meier survival studies were performed with AZD8055 and in combination with TMZ.

Results: We confirm that gefitinib and rapamycin have modest effects in most BTIC lines, but AZD8055 was highly effective at inhibiting Akt/mTORC2 activity and dramatically reduced the viability of BTICs regardless of their EGFR and PTEN mutational status. Systemic administration of AZD8055 effectively inhibited tumor growth in subcutaneous BTIC xenografts and mTORC1/2 signaling in orthotopic BTIC xenografts. AZD8055 was synergistic with the alkylating agent TMZ and significantly prolonged animal survival.

Conclusion: These data suggest that dual inhibition of mTORC1/2 may be of benefit in GBM, including the subset of TMZ-resistant GBMs.

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Introduction

Glioblastoma (GBM) is an aggressive brain tumor that is poorly controlled with currently available treatments. The alkylating agent temozolomide (TMZ) is the first-line chemotherapeutic agent used in GBM and is effective against tumors lacking expression of the O6-methylguanine methyltransferase (MGMT) enzyme due to promoter methylation (1). However, MGMT-methylated tumors eventually become resistant to TMZ, and there is a clear need for effective second-line agents for patients with GBM. As such, translation of our rapidly expanding knowledge of the molecular biology of GBM, together with the growing repertoire of molecular-based chemotherapeutics now available, has the potential to dramatically improve patient outcomes.

The Cancer Genome Atlas (TCGA) implicates aberrations in growth factor signaling in 88% of GBM tumors (2). Alterations to the EGFR gene are particularly common with 40% of GBMs harboring EGFR amplifications or the constitutively active EGFRvIII mutation (2–4). Aberrations in downstream effector pathways of receptor tyrosine kinases (RTK) are also common in GBM, with mutation, silencing, or deletion of PTEN, the endogenous regulator of PI3K signaling, being the most frequent alteration (occurring in 36% of GBMs; ref. 2). Mutations of EGFR and PTEN frequently coincide in GBM and have been hypothesized to act in synergy to deregulate growth factor signaling networks (2, 5–7).
Translational Relevance

Temozolomide (TMZ) chemotherapy is the standard-of-care for glioblastoma (GBM), but only affords increased overall survival in the minority of patients with MGMT-methylated tumors and nearly all GBMs eventually become TMZ resistant. Effective second-generation chemotherapeutics, thus, are clearly needed, especially for the majority of tumors that are MGMT unmethylated. Molecular therapeutics targeting the PI3K/mTORC1/2 pathway hold promise in GBM treatment. We demonstrate that dual mTORC1/2 inhibition with AZD8055 was highly effective both in vitro and in vivo at decreasing proliferation and inducing apoptosis in a diverse panel of patient-derived brain tumor–initiating cells (BTIC). Furthermore, daily oral coadministration of AZD8055 with TMZ was well tolerated and had a striking synergistic effect on increasing median survival in orthotopic xenografts of BTICs with unmethylated MGMT promoters. Dual mTORC1/2 inhibitors in combination with TMZ, thus, have the potential for translation into a broadly effective therapeutic strategy for patients with GBM.

With the prevalence of EGFR and PTEN mutations in GBM, a significant focus over the last several decades has centered on the development of molecular therapeutics targeted to these aberrations. In particular, EGFRvIII has garnered significant attention as a promising drug target, but EGFR inhibitors such as gefitinib and erlotinib have failed to demonstrate more than modest results in most patients (8–9). Similarly, inhibition of PI3K/mTORC1 with rapamycin or related analogs has achieved only modest benefit in small patient subgroups (10, 11). Hence, the results of these past clinical trials (8–11, 12) suggest that therapeutic targeting of EGFR or PI3K signaling is inhibited through mTORC1 but not mTORC2 (reviewed in ref. 14). Recently, the small-molecule inhibitor AZD was in combination with TMZ, thus, have the potential for translation into a broadly effective therapeutic strategy for patients with GBM.

EGFR and PTEN sequencing

RNA was extracted from fresh GBM tissue and sphere cultures using the RNeasy Kit (Qiagen) according to the manufacturer’s instructions. Of note, 500 ng of RNA was reverse transcribed with the Superscript III First-Strand Synthesis System (Invitrogen) using oligo-dT primers. In addition, 2 μL of cDNA was used in a 50 μL RT-PCR reaction spectrum characteristic of GBM (16, 17). Our group has successfully established a large collection of BTIC lines from patients with GBM that display the molecular heterogeneity of the disease, including, to our knowledge, the first reported lines endogenously expressing EGFRvIII in vitro and in orthotopic xenografts (18–20). Here, we use subgroups of BTICs with different combinations of EGFR and PTEN alterations as a model system to interrogate the molecular characteristics that influence response to EGFR and mTORC1/2 inhibition. We report that inhibition of EGFR with gefitinib and mTORC1 with rapamycin was only modestly effective in most BTIC lines tested and the combination of gefitinib with rapamycin was moderately synergistic in EGFRvIII/PTENmt BTICs. In contrast, dual inhibition of mTORC1/2 with AZD significantly reduced cell viability in all BTIC lines tested regardless of their underlying EGFR and PTEN mutational status. Furthermore, AZD was synergistic with TMZ in MGMT-unmethylated BTICs. Systemic administration of AZD was tolerated by mice and significantly reduced subcutaneous BTIC tumor growth, inhibited intracranial mTORC1/2 signaling, and induced apoptosis in orthotopic BTIC xenografts. Finally, combinatorial treatment of AZD and TMZ significantly increased survival in mice orthotopically xenografted with an aggressive EGFRvIII, PTEN-mutant, MGMT-unmethylated BTIC line from a TMZ-resistant recurrent GBM.

Materials and Methods

Brain tumor sphere culture

GBM BTICs were cultured from tumor specimens from adult patients with GBM during their operative procedure, as previously described (18, 20), following informed consent and approval by the University of Calgary Ethics Review Board. Normal adult human brain cells were cultured from a sample of white matter obtained from a 16-year-old female undergoing a corpus callosotomy for drug-resistant epilepsy. The tissue was enzymatically dissociated as previously described for GBM tumor tissue (18) and cultured as an adherent monolayer in BTIC culture medium with 10% FBS. BTICs were orthotopically xenografted in NOD/SCID mice as previously described (18). Human fetal neural stem cells were also cultured as previously described (21) and induced to differentiate into astrocytes by addition of 10% FBS and removal of EGF, FGF-2, and heparin sulfate. Authentication and testing of all cell lines was performed as per American Association for Cancer Research recommendations. Molecular characterization of all BTIC lines was performed as described below at initiation and before experimental use.

EGFR and PTEN sequencing

RNA was extracted from fresh GBM tissue and sphere cultures using the RNeasy Kit (Qiagen) according to the manufacturer’s instructions. Of note, 500 ng of RNA was reverse transcribed with the Superscript III First-Strand Synthesis System (Invitrogen) using oligo-dT primers. In addition, 2 μL of cDNA was used in a 50 μL RT-PCR reaction
fragments (fragment 1: forward: 5'-GTG GC -3'
reverse: 5'-TGT-3'); fragment 2: forward: 5'-CTC CAC ATC CTG CCG GTG GC-3'; reverse: 5'-GCA CCA AGC GAC GGT CCT CT-3'; fragment 3: forward: 5'-CTG GTG GTG GCC CTG GGG AT-3'; reverse: 5'-GCG GGC ATG GTT GGG AT-3'). Each fragment was then sequenced bidirectionally (fragment 1: forward strand: 5'-AGC TCT TCG GGG AGC AGC GA-3', reverse strand: 5'-AGG CAT GGA GGT CCG TCC TGT-3'; fragment 2: forward strand: 5'-GGG GTG ACT CCT GGA TCT GCA CAC ACC -3'; fragment 3: forward strand: 5'-CCA GCG TGG ACA ACC CCC AC-3', reverse strand: 5'-TGC TGT GGC GTG TTC CTG GG-3'). The RT-PCR products were purified by agarose gel electrophoresis and isolated with the QiAquick Gel Extraction Kit (Qiagen). Automated DNA sequencing was performed at the University of Calgary Core DNA Services facility. Expression of EGFRvIII was also confirmed by RT-PCR as previously described (9).

MGMT promoter methylation assay

Of note, 500 ng of DNA was bisulfite converted with the Epitect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. In addition, 2 µL of each Epitect product was used for methylation-specific PCR (MS-PCR) determination of MGMT promoter methylation as previously described (1). Thermocycling conditions for MGMT MS-PCR were 95°C for 10 minutes initial denaturation, then 35 cycles of 95°C for 45 seconds denaturation, 45 seconds annealing, 72°C for 45 seconds extension, and a final 10 minutes extension at 72°C. The annealing temperatures were 61°C for MGMT-methylated and 58°C for MGMT-unmethylated MS-PCR.

Western blotting

BTIC spheres were lysed in modified RIPA buffer supplemented with Complete Mini protease (Roche) and Halt phosphatase (Thermo Scientific) inhibitor cocktails. For protein analysis following drug treatment, BTIC spheres were dissociated to single cells and 1 x 10^6 cells were treated with gefitinib (LC Laboratories), rapamycin (Calbiochem), AZD8055 (Selleck Chemicals), TMZ (Merck), or vehicle (DMSO) for 2 or 24 hours. Of note, 15 µg of protein was loaded on 7.5% or 10% SDS-PAGE gels and blotted to nitrocellulose membranes. Blots were stained with the following antibodies: phospho-EGFR Y1068 (1.0 µg/mL; R&D Systems), EGFR (1:1,000; Cell Signaling Technology), phospho-Erk1/2 T202/Y204 (1:1,000; Cell Signaling Technology), Erk1/2 (1:5,000; Millipore), phospho-Akt S473 (1:1,000; Cell Signaling Technology), Akt (1:1,000; Cell Signaling Technology), phospho-S6 S240/244 (1:1,000; Cell Signaling Technology), phospho-4E-BP1 S65 (1:1,000; Cell Signaling Technology), and actin (1:2,500; Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies (donkey anti-mouse, donkey anti-goat, and goat anti-rabbit; Calbiochem) were used at 1:6,000. Bands were visualized with the ECL Plus Western Blotting Detection System and Hyperfilm (Amersham).

BTIC growth assays

Dissociated BTIC spheres were seeded at 1,500 cells per well and treated with gefitinib, rapamycin, AZD, TMZ, or DMSO 1 day after plating. Cell viability following drug treatment was assessed 8 days later using the alamarBlue assay (Invitrogen) according to the manufacturer’s instructions. Drug sensitivity was also assessed using the neurosphere assay in which 500 to 2,500 cells were seeded per 96-well, treated with gefitinib, rapamycin, or DMSO, and the number of spheres counted 14 to 28 days later. All culture experiments were performed in triplicate with a minimum of 3 wells per condition.

Subcutaneous BTIC xenografts

BTIC73 spheres were dissociated to single-cell suspensions, and 1 x 10^5 cells were implanted into the subcutaneous tissue of the left flank of 6- to 8-week-old NOD/SCID mice. Eighteen days after BTIC implantation, mice were randomized to vehicle or treatment cohorts. AZD8055 (15 mg/kg) or vehicle (40% PEG300 in dH2O; Sigma-Aldrich) was injected i.p. on days 18, 20, 22, 25, 27, and 29. Two hours after the final injection on day 29, the mice were euthanized with sodium pentobarbital. The subcutaneous tumors were dissected out of the flank, weighed, fixed in formalin, and prepared for histology and IHC.

Intracranial BTIC xenografts

BTIC147 spheres were dissociated to single-cell suspensions, and 1 x 10^4 cells were stereotactically implanted into the right striata of 6- to 8-week-old NOD/SCID mice as previously described (18, 23). Fourteen days after BTIC implantation, mice were randomized to vehicle or treatment cohorts. AZD8055 (10 mg/kg) or vehicle (40% PEG300 in dH2O; Sigma-Aldrich) was injected i.p. on days 14, 15, 16, 19, 20, 21, and 22. Two hours after the final injection on day 22, the mice were euthanized with sodium pentobarbital followed by transcardiac perfusion with 4% paraformaldehyde. Brains were removed, fixed in formalin, and prepared for histology and IHC. For Kaplan–Meier survival studies, 5 x 10^4 BTIC147 or BTIC206 cells were orthotopically xenografted in the brains of NOD/SCID mice. Five days after BTIC implantation, mice were randomized to vehicle or treatment cohorts. Vehicle (Captisol; Captisol Ligand Technology and Ora-Plus), AZD8055 (2.5, 5, and 10 mg/kg resuspended in Captisol), TMZ (50 mg/kg week 1, 10 mg/kg weeks 2 and 3; Sigma-Aldrich), or combinations were delivered orally 5 days a week for 3 weeks. Mice were sacrificed upon significant weight loss or presentation of neurologic symptoms necessitating euthanasia as per University of Calgary animal care guidelines. Body weights were recorded every other day starting at day 1 of the treatment regimen until the end of the treatment period and at 1 week after the last treatment. Necropsy and cranial dissection were performed to confirm presence of tumor in all animals.
**Quantification of AZD by liquid chromatography/mass spectrometry**

Cohorts of 3 NOD/SCID mice were treated with a single oral dose of AZD8055 at 2.5 mg/kg, 5 mg/kg, or 10 mg/kg. Blood samples were obtained 30 and 300 minutes later and centrifuged to prepare plasma. At 300 minutes, mice were euthanized and brain samples were also obtained. All samples were stored at −20°C until analysis by LC/MS.

**Immunohistochemistry**

Xenograft sections were immunostained for human nucleolin (1:1,000; Millipore), phospho-S6 S240/244 (1:100; Cell Signaling Technology), phospho-Akt S473 (1:100; Cell Signaling Technology), total Akt (1:100; Cell Signaling Technology), and phospho-NDRG1 T346 (1:100; Cell Signaling Technology). Staining was visualized with Vectastain Elite mouse IgG or rabbit IgG ABC kits (Vector Laboratories) and DAB (3,3'-diaminobenzidine) substrate (Sigma-Aldrich), followed by hematoxylin counterstaining. Xenograft tumor cells undergoing apoptosis were detected with the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International) according to the manufacturer’s instructions.

**Statistical analysis**

Error bars represent SD and asterisks denote statistically significant differences from vehicle-treated wells determined by the Student t test. ANOVA and the Tukey multiple comparison test (α = 0.05) were performed to assess synergy of concurrent gefitinib and rapamycin, gefitinib and AZD, and AZD and TMZ. A Mann–Whitney test was used to assess the effect of AZD on subcutaneous tumor weight. Two-way ANOVA was used to assess differences in body weights of animals in different treatment cohorts. Statistically significant differences in median survival were determined by the log-rank test using GraphPad Prism.

**Results**

**BTICs provide a unique model of endogenous EGFR- and PTEN-mutant glioma**

To assemble a panel of BTIC lines representative of the potential combinations of EGFR and PTEN mutational status, we sequenced the entire EGFR and PTEN open reading frames. Five lines demonstrated EGFRvIII expression by RT-PCR, cDNA sequencing, and Western blotting (Supplementary Fig. S1A, S1B, and S1D; Table 1). Diverse point mutations and frameshift insertions and deletions in PTEN were also detected in five BTIC lines (Supplementary Fig. S1C; Table 1). Expression of full-length PTEN protein was confirmed by Western blot analysis in all PTENwt and point mutant BTICs (Supplementary Fig. S1D). Western blotting also confirmed an absence of full-length PTEN protein in frameshift mutant lines except for BTIC12, which harbors a frameshift alteration near the C-terminus. We selected a panel of 13 BTIC lines that spanned the spectrum of potential combinations of EGFR and PTEN mutations as a model system to evaluate sensitivity to EGFR and PI3K/mTORC1/2 blockade in relation to EGFR and PTEN status (Table 1). Moreover, although expression analyses would be needed for definitive subtyping characterization, it is likely that at the very least, this BTIC panel encompasses lines derived from the classical and mesenchymal TCGA GBM subgroups.

**Table 1. Identification of a panel of BTICs representative of the combinations of EGFR and PTEN mutations that exist in GBM.**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Sex</th>
<th>EGFR status</th>
<th>PTEN status</th>
<th>MGMT status</th>
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<td>M</td>
</tr>
<tr>
<td>12</td>
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<td>59</td>
<td>M</td>
<td>WT</td>
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<tr>
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<tr>
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<td>WT</td>
<td>V341F</td>
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<tr>
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NOTE: GBM-r, samples obtained from tumors at recurrence following initiation of radiation and temozolomide; M and U, samples with methylated and unmethylated MGMT promoters, respectively.
Gefitinib modestly inhibits growth and survival of PTENwt and EGFRvIII BTICs.

Gefitinib sensitivity was determined for the four BTIC molecular subgroups by assessing cell viability with alamarBlue. Low micromolar concentrations of gefitinib reduced viability of EGFRvIII/PTENwt BTICs in a dose-dependent manner, but had minimal effect on EGFRwt/PTENmt BTICs or normal adult human brain cells (Fig. 1A). A concentration of 2 μmol/L of gefitinib efficiently inhibited EGFR phosphorylation after 2 hours (Fig. 1B) and was used to screen our entire panel of BTIC lines. PTENwt BTIC lines were modestly sensitive to gefitinib regardless of EGFR status, whereas only one EGFRvIII/PTENmt line (BTIC103) and no EGFRwt/PTENmt lines were sensitive to gefitinib (Fig. 1C and Supplementary Fig. S2A). Congruent results were also obtained with the neurosphere assay; EGFRvIII/PTENwt BTICs formed fewer neurospheres (P < 0.001) with the addition of gefitinib as compared with vehicle, whereas the number of spheres in EGFRwt/PTENmt BTICs was only modestly reduced (P < 0.001. Supplementary Fig. S2B). Western blotting for downstream signaling effectors revealed that gefitinib dramatically reduced EGFR and Erk1/2 phosphorylation in gefitinib-sensitive EGFRvIII/PTENwt BTICs, but did not affect phosphorylation of Akt and S6 (Fig. 1D). Taken together, these data indicate that gefitinib is only able to achieve modest effects in most BTIC lines and blockade of EGFR is insufficient to inhibit mTORC1/2 activity.

mTORC1 blockade with rapamycin partially inhibits growth of BTICs.

Given the association of gefitinib sensitivity with wild-type PTEN status and the consistent inability of gefitinib to inhibit downstream PI3K signaling in EGFRvIII BTICs, we next investigated mTORC1 inhibition with rapamycin. At a concentration of 0.1 μmol/L rapamycin, both PTENwt and PTENmt BTICs demonstrated a partial reduction in cell viability, whereas normal adult human brain cells remained unaffected (Fig. 1A). Western blotting confirmed that 0.1 μmol/L rapamycin effectively reduced phosphorylation of the mTORC1 effectors S6 and 4E-BP1 (Fig. 1B). Cell viability was significantly but not fully reduced by 0.1 μmol/L rapamycin in all BTICs except for one EGFRvIII/PTENmt BTIC (Fig. 1C and Supplementary Fig. S3A). Concordant results were also observed using the neurosphere assay with reduced sphere numbers in both EGFRvIII/PTENwt and EGFRwt/PTENmt BTICs (Supplementary Fig. S3B). Inhibition of mTORC1 activity, therefore, appears to only have a partial effect on reducing BTIC growth.

Concurrent EGFR and mTORC1 inhibition is synergistic in EGFRvIII/PTENwt BTICs.

We next investigated concurrent gefitinib and rapamycin as a means to synergistically augment drug response by concurrent inhibition of downstream effectors of the EGFR and PI3K/mTOR pathways. In EGFRvIII/PTENmt lines, concurrent gefitinib and rapamycin resulted in a significantly greater, although still partial, reduction in cell viability as compared with either agent alone (P < 0.05 for both gefitinib alone and rapamycin alone vs. concurrent gefitinib and rapamycin. Tukey multiple comparison test; Fig. 1C). BTICs from the three other molecular subgroups all failed to show a response to concurrent gefitinib and rapamycin, had a summative effect on reducing phosphorylation of the mTORC1 effectors S6/4E-BP1 in EGFRvIII/PTENmt BTICs (Fig. 1D). However, concurrent gefitinib and rapamycin appeared unable to inhibit mTORC2 activity as minimal change in the phosphorylation of Akt S473 was seen (Fig. 1D). Together, these data suggest that the combination of gefitinib and rapamycin has complementary effects on downstream signaling, but only achieved a modestly synergistic effect in EGFRvIII/PTENwt BTICs.

Combined mTORC1/2 inhibition dramatically decreases viability of BTICs regardless of EGFR and PTEN status.

Given the persistent levels of Akt activity in BTICs despite gefitinib and rapamycin treatment, we next investigated AZD8055, a small-molecule inhibitor capable of blocking both mTORC1 and mTORC2 activity. At a concentration of 2 μmol/L, AZD dramatically reduced alamarBlue conversion in both PTENwt and PTENmt BTICs, but did not affect normal human astrocytes (Fig. 2A). All BTIC lines in our panel demonstrated a dramatic response to 2 μmol/L AZD, regardless of their underlying EGFR or PTEN status (Fig. 2B). Western blotting confirmed that 2 μmol/L AZD effectively reduced phosphorylation of Akt, S6, and 4E-BP1 demonstrating near total inhibition of both mTORC1 and mTORC2 (Fig. 2C). The combination of gefitinib and AZD also reduced Erk1/2 phosphorylation, but did not further reduce BTIC viability below that achieved with AZD alone (data not shown). These data suggest that mTORC2 activity is crucial for BTIC growth and combined inhibition of mTORC1/2 with AZD dramatically decreases BTIC viability.

Combined mTORC1/2 inhibition enhances response to TMZ in MGMT-unmethylated BTICs.

Deregulation of the PI3K pathway through PTEN deficiency has been shown to increase MGMT expression and consequently confer enhanced resistance to TMZ in a mouse model of glioma (24). Moreover, activation of mTORC2 and downstream NFκB signaling has recently been demonstrated to be a novel mechanism underlying chemoresistance to cisplatin in GBM cell lines (25). Therefore, we next investigated the combination of AZD with TMZ, the current standard-of-care and only chemotherapeutic agent widely used for all patients with GBM, as a means to augment therapeutic response in GBM. Across our entire panel of BTICs, we found that AZD was compatible with TMZ because in no case was the
Figure 1. Gefitinib and rapamycin have modest effects on BTICs. A, gefitinib reduced cell viability in EGFRvIII/PTENwt BTICs, but not EGFRwt/PTENmt BTICs or normal adult human brain cells in a dose-dependent manner at low micromolar concentrations. Similarly, rapamycin reduced cell viability in both EGFRvIII/PTENwt and EGFRwt/PTENmt BTICs in a dose-dependent manner. B, Tyr1068 EGFR phosphorylation was reduced after 2 hours of exposure to 2 μmol/L gefitinib, whereas phosphorylation of S6 and 4E-BP1 was dramatically reduced after 2 hours of exposure to 0.1 μmol/L rapamycin. C, gefitinib and rapamycin modestly reduced cell viability in most BTIC lines. The combination of gefitinib and rapamycin was synergistic in EGFRvIII/PTENmt BTICs, but not BTICs from any of the three other molecular subgroups (*, P < 0.05; Tukey multiple comparison test). D, gefitinib markedly reduced phosphorylation of EGFR tyrosine 1068 and Erk1/2, whereas rapamycin reduced phosphorylation of S6 and 4E-BP1. Phosphorylation of Akt was unaffected by either drug applied singly or in combination.
combination of AZD and TMZ less effective than TMZ alone (Fig. 3A). However, in MGMT-unmethylated lines, AZD alone or in combination with TMZ achieved a dramatically larger reduction in BTIC viability than that achieved with TMZ alone. AZD also appeared to act synergistically with TMZ at lower doses that did not maximally reduce BTIC viability on their own. The lower dose of 0.01 μmol/L AZD in combination with TMZ was significantly more effective than either agent alone in decreasing alamarBlue conversion and BTIC sphere formation in MGMT-unmethylated lines (Fig. 3B–C). Moreover, the combination of 0.01 μmol/L AZD with TMZ was able to substantially increase cleavage of the apoptotic marker PARP to levels comparable with that achieved with 2 μmol/L AZD (Fig. 3D). Taken together, these data suggest that AZD can be combined with TMZ and may act synergistically in MGMT-unmethylated BTICs.

Systemic administration of AZD reduces tumor growth, inhibits proliferation, and induces apoptosis in vivo in subcutaneous BTIC xenografts

We next evaluated the efficacy of AZD in preclinical xenograft models. NOD/SCID mice bearing established, externally apparent, subcutaneous BTIC xenograft tumors were treated with thrice weekly i.p. injections of AZD. After 2 weeks, this regimen resulted in an obvious reduction in gross tumor extent (Fig. 4A) and statistically significant reduction in tumor weight (Fig. 4B). On hematoxylin and eosin (H&E) staining, the tumors in vehicle-treated animals were densely cellular with frequent mitotic figures whereas the tumors in AZD-treated animals had few mitotic figures and large areas of geographic necrosis (Fig. 4C). Moreover, immunostaining for the proliferative marker Ki67 was decreased whereas TUNEL reactivity was increased in the treated xenografts compared with vehicle controls,
confirming that AZD effectively induced cytostasis and apoptosis in tumor cells (Fig. 4C).

**Systemic administration of AZD inhibits intracranial mTORC1/2 signaling, induces apoptosis, and prolongs animal survival in combination with TMZ in orthotopic BTIC xenografts**

To further evaluate the efficacy of AZD in a more clinically relevant model, NOD/SCID mice bearing intracranial BTIC xenograft tumors were treated with i.p. injections of AZD 14 days after implantation to allow formation of a tumor mass. The series of two sets of three daily injections with a 2-day break in between were well tolerated without unacceptable side effects. Staining with an antibody specific for human nucleolin confirmed tumor establishment (Fig. 5A–B). Histologic analysis of the xenograft tumors confirmed that AZD effectively inhibited intracranial mTORC1/2 signaling in vivo as evidenced by dramatic reductions in phosphorylation of the mTORC1 downstream effector S6 and the mTORC2 effectors Akt S473 and NDRG1. Administration of AZD did not decrease Ki67 immunostaining (data not shown) as seen in the subcutaneous xenografts, but did dramatically increase TUNEL staining, thus confirming induction of apoptosis in intracranial xenograft tumor cells (Fig. 5A–B).

We next asked whether AZD treatment would increase survival in mice orthotopically xenografted with BTICs. To achieve a more clinically relevant model, we administered AZD orally five times a week for 3 weeks. AZD was well
 tolerated by all animals (Supplementary Fig. S4), but did not significantly improve survival in three different BTIC lines [73 (data not shown), 147 (Fig. 5C) and 206 (Supplementary Fig. S5)]. LC/MS analysis of plasma and brain samples collected 30 and 300 minutes after administration of AZD showed rapid plasma clearance with a peak AZD concentration of only 100 nm detected in the brain (data not shown). Thus, although daily systemic administration of AZD is capable of effectively inhibiting intracranial mTORC1/2 signaling and inducing apoptosis in BTICs in vivo, it appears unable to achieve the sustained intracranial concentrations necessary to increase animal survival by itself. Careful optimization of the dosing regimen and enhancement of blood–brain barrier (BBB) permeability of AZD or other similar dual mTORC1/2 inhibitors is, thus, necessary to achieve efficacy in orthotopic xenografts and facilitate clinical translation.

Given the synergy of AZD8055 with TMZ observed in vitro in MGMT-unmethylated BTIC lines, we asked whether a combinatorial approach might be effective in vivo. NOD/SCID mice bearing intracranial BTIC xenografts were treated with AZD or TMZ alone and in combination. BTIC206 xenografts (MGMT-methylated/EGFRwt/PTENwt) were highly responsive to TMZ alone with all animals surviving to the predetermined experiment end-point of 50 days compared with a median survival of 60 days in the AZD alone and TMZ alone cohorts (Fig. 5C, P < 0.0001; log-rank test). These data, thus, confirm that the addition of AZD to TMZ is synergistic in vivo and holds considerable promise for clinical translation, especially in MGMT-unmethylated and recurrent GBMs resistant to TMZ.

Discussion

Although numerous preclinical studies, including the recent TCGA results, have strongly implicated aberrant EGFR and PI3K signaling in the biology of GBM, this knowledge has yet to translate into new effective treatments for patients. The results of recent clinical trials for gefitinib and other small-molecule EGFR inhibitors suggest that EGFR blockade has limited efficacy, except in specific patient subgroups with particular combinations of mutations (8, 9). Residual PI3K pathway activity following EGFR blockade has been hypothesized to be a major factor underlying resistance to gefitinib (9) and concurrent EGFR and mTOR inhibition has been reported to increase sensitivity to EGFR inhibition in vitro (13). However, mTORC1 blockade with rapamycin or concurrent gefitinib and rapamycin have yielded disappointing results in clinical trials, suggesting that these approaches are still likely to be inadequate for most patients (12). Our results support these clinical findings by demonstrating that gefitinib and rapamycin are only modestly effective, regardless of the underlying EGFR and
PTEN mutational status. The inability of gefitinib and rapamycin to fully inhibit the potent prosurvival and proliferative signaling of Akt, that we (Supplementary Fig. S6A) and others (ref. 25; reviewed in ref. 14) have observed, seems to explain the disappointing results in patients with brain tumor. Moreover, the mutational status of EGFR and PTEN alone did not clearly predict which of our BTIC lines would respond to gefitinib or rapamycin. This further complicates the rational use of EGFR and mTORC1 blockade, as simple molecular tests would seem unlikely to be able to identify the small minority of patients responsive to gefitinib and rapamycin.

Our data with AZD strongly support the hypothesis that combined blockade of mTORC1 and mTORC2 is a potent strategy for inhibiting BTICs both in vitro and in vivo without an apparent need for simultaneous inhibition of EGFR or other RTKs. In agreement with our findings, the dual mTORC1/2 inhibitors CC214-1 and CC214-2 have been recently reported to effectively inhibit growth of GBM cell lines and xenografts resistant to mTORC1 blockade with rapamycin (26). These results support the idea that inhibition of mTORC2 must be achieved for effective blockade of PI3K signaling (Supplementary Fig. S6B). Moreover, all BTIC lines that we tested were sensitive to AZD, at concentrations that did not significantly affect the viability of normal human astrocytes. Preestablished tumors from subcutaneous xenografts of an aggressive EGFRvIII/PTEN-mutant cell line were dramatically reduced in mass and demonstrated decreased proliferation and increased apoptosis upon AZD administration (Fig. 4). Furthermore, administration of AZD inhibited intracranial xenograft mTORC1/2 signaling in mice, with clear reduction in pAkt S473 activation and increased apoptosis (Fig. 5). Although longer-term administration of AZD alone in the orthotopic model did not significantly increase animal survival, this likely was a result of this particular compound having insufficient BBB permeability to achieve sustained intracranial concentrations with a single daily oral dose. In our subcutaneous xenograft model, thrice-weekly AZD was quite effective at reducing tumor volume, inhibiting proliferation, and inducing apoptosis. Taken together, these data strongly suggest that dual mTORC1/2 inhibitors, would be clinically effective with the proper pharmacokinetic profiles necessary for maximizing blood-brain barrier permeability to achieve sustained target inhibition while maintaining limited systemic toxicity on prolonged treatment.

Figure 5. Systemic administration of AZD inhibits intracranial mTORC1/2 signaling leading to apoptosis of tumor cells and synergizes with TMZ. A and B, staining with an antibody specific for the human homolog of nucleolin demonstrated the presence of a BTIC xenograft tumor in the deep gray matter. AZD dramatically decreased phosphorylation of S6 in treated animals demonstrating on-target inhibition of mTORC1 signaling. Phosphorylation of the S473 residue of Akt and the T346 residue of NDRG1 was decreased without affecting levels of total Akt, thus demonstrating on-target inhibition of mTORC2 signaling. Arrowheads, xenograft cells with cytoplasmic staining for phosphorylated NDRG1. AZD treatment resulted in increased staining with TUNEL demonstrating apoptosis in tumor cells following mTORC1/2 inhibitors in vivo. C, oral administration of AZD and TMZ significantly increased median survival of BTIC147 xenograft animals as compared with that with TMZ alone or AZD alone (78 vs. 60 days; P < 0.0001; log-rank test).
Although Gini and colleagues (26) reported that ectopic expression of the EGFRvIII mutation or PTEN deficiency was necessary to sensitize U87 cells to dual mTORC1/2 inhibition with CC214-2, we found that all BTICs we tested were sensitive to AZD regardless of their underlying endogenous EGFR and PTEN mutational status. Therefore, mTORC1/2 signaling may be a key signaling hub, common to the biology of molecularly diverse GBMs, and provides support for clinical use of mTORC1/2 inhibitors. Moreover, the near universal sensitivity of BTICs to AZD suggests that preselection of patients based on tumor mutation analyses may not be necessary, thus streamlining potential clinical translation of dual mTORC1/2 inhibitors.

In addition to reducing BTIC viability on its own, we also found that AZD was compatible with TMZ chemotherapy. TMZ is the standard-of-care and only chemotherapeutic agent routinely used for all patients with GBM. However, TMZ has only been demonstrated to afford a significant benefit in overall survival (OS) to the minority of patients with GBM whose tumors are methylated at the MGMT promoter (1). Conversely, in the majority of patients whose tumors are MGMT unmethylated, TMZ affords only a modest and statistically insignificant increase in OS (1). Hence, additional chemotherapeutic agents that are broadly effective in patients with GBM, especially in the large cohort of MGMT-unmethylated patients, are clearly needed. The data that we present here suggest that mTORC1/2 inhibitors have the potential to fill this role as an effective second-line chemotherapeutic strategy to complement TMZ. We found that in MGMT-methylated lines, AZD was equally, if not more effective than TMZ at reducing BTIC viability and in no case did concurrent addition of TMZ with AZD decrease sensitivity to TMZ in vitro or in vivo. The efficacy of AZD was even more dramatic in MGMT-unmethylated BTICs, including several lines isolated from highly TMZ-resistant tumors at recurrence, in which 2 μmol/L AZD was able to reduce BTIC viability by 90% or more. Even more strikingly, low nanomolar concentrations of AZD demonstrated synergy with TMZ in MGMT-unmethylated BTICs, and thus may provide a feasible means of enhancing response to TMZ in this subgroup of GBM, for which few effective therapeutic options are currently available. PI3K signaling is known to have a role in drug resistance, as Akt has recently been shown to regulate activity of the ABCG2 drug transporter protein in glioma stem cells (24). Although TMZ does not appear to be a direct substrate for ABCG2, Bleau and colleagues (24) report that TMZ selects for the side population of stem-like glioma cells, especially when deficient for PTEN. Moreover, siRNA knockdown of the key mTORC2 component Rictor was recently shown to dramatically reverse resistance of EGFRvIII-expressing GBM cell lines to cisplatin (25). As such, effective mTORC1/2 inhibition with AZD may be able to eradicate the TMZ-resistant cell subpopulation within GBM.

Our data clearly show that sustainable mTORC1/2 inhibition holds promising therapeutic potential for GBM treatment, without a need for direct inhibition of upstream receptors such as EGFR. Moreover, the combination of AZD and TMZ may be particularly useful as an effective second-line salvage option for patients who have become resistant to TMZ, especially in tumors with unmethylated MGMT promoters. Indeed, this was clearly demonstrated by the striking increase in survival with the combination of AZD and TMZ in our orthotopic xenograft model of BT147, an MGMT-unmethylated/EGFR vIII/PTEN mt BTIC line derived from a TMZ-resistant recurrent GBM. Our data strongly support the premise that clinical efficacy in the management of GBM may be achievable if both mTORC1/2 complexes are effectively targeted in conjunction with TMZ chemotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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