A Dual Phosphoinositide-3-Kinase α/mTOR Inhibitor Cooperates with Blockade of Epidermal Growth Factor Receptor in *PTEN*-Mutant Glioma

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Abstract

We have shown previously that blockade of epidermal growth factor receptor (EGFR) cooperates with a pan-selective inhibitor of phosphoinositide-3-kinase (PI3K) in *EGFR*-driven glioma. In this communication, we tested *EGFR*-driven glioma differing in *PTEN* status, treating with the EGFR inhibitor erlotinib and a novel dual inhibitor of PI3Kα and mTOR (PI-103). Erlotinib blocked proliferation only in $PTEN^{wt}$ cells expressing EGFR. Although erlotinib monotherapy showed little effect in $PTEN^{mt}$ glioma, PI-103 greatly augmented the antiproliferative efficacy of erlotinib in this setting. To address the importance of PI3K blockade, we showed in $PTEN^{mt}$ glioma that combining PI-103 and erlotinib was superior to either monotherapy or to therapy combining erlotinib with either rapamycin (an inhibitor of mTOR) or PIK-90 (an inhibitor of PI3Kα). These experiments show that a dual inhibitor of PI3Kα and mTOR augments the activity of EGFR blockade, offering a mechanistic rationale for targeting EGFR, PI3Kα, and mTOR in the treatment of EGFR-driven, *PTEN*-mutant glioma. [Cancer Res 2007;67(17):7960–5]

Introduction

Both amplification of epidermal growth factor receptor (EGFR) and activation of phosphoinositide-3-kinase (PI3K) feature prominently in glioma (1). Activation of PI3K that occurs as a consequence of *EGFR* amplification should respond to inhibitors of EGFR. PI3K may also be activated independently of EGFR through gain-of-function mutations in PI3K or from inactivation of the lipid phosphatase *PTEN*, a negative regulator of PI3K (2–4). Because the activation of PI3K is uncoupled from the upstream amplification of *EGFR* in *PTEN*-mutant glioma, we previously tested inhibitors of PI3K and of EGFR in combination, demonstrating the efficacy for this approach in *EGFR*-driven, *PTEN*-mutant glioma (5). The clinical relevance of this work was recently corroborated. In two retrospective studies, tumors in which PI3K was activated independently of EGFR responded poorly to EGFR inhibition (6, 7). Collectively, these reports suggest that in tumors with *EGFR* amplification and *PTEN* inactivation (comprising half of *EGFR*-amplified glioma), combined inhibition of EGFR and PI3K represents a promising therapy.

Toxicity associated with pan-selective inhibition of PI3Ks is a major hurdle preventing the translation of this approach to patients (8). To assess the impact of inhibiting individual PI3Ks, we recently synthesized a series of isoform-selective inhibitors of PI3Ks and enumerated their biochemical targets (9, 10). Using this chemical array, we linked increased specificity to decreased toxicity for agents targeting within the PI3K family and identified PI3Kα as critical for proliferation in malignant glioma (11). We noted particular efficacy in combining inhibitors of PI3Kα with inhibitors of mTOR, a serine-threonine kinase complex critical for cell growth (12). This result was at first somewhat surprising because mTOR is activated in response to PI3K. PI3Ks activate the pleckstrin homology domain containing serine-threonine kinase Akt. Akt, in turn, signals through effectors, including mTOR, to promote viability (13). Importantly, inhibitors of mTORC1 actually activate signaling through PI3K (11, 14, 15). Thus, the efficacy of mTOR blockade is achieved at the cost of driving other outputs of Akt signaling, contributing to the overall disappointing results observed using inhibitors of mTOR clinically. Combined blockade of mTOR and PI3Kα shuts down mTOR and, in addition, abrogates the activation of PI3K observed using mTOR inhibitors alone. We recently validated this dual-inhibitor approach by combining rapamycin, an inhibitor of mTOR, with PIK-90, an inhibitor of PI3Kα, and also through testing a dual inhibitor of PI3Kα and of mTOR (PI-103) that was well tolerated and highly effective against glioma xenografts (11).

These preclinical studies support the use of combination therapy directed against EGFR and PI3K and, in parallel, show that inhibitors of PI3Kα cooperate with inhibitors of mTOR in glioma. The goal of our current work is to test inhibitors of EGFR in combination with inhibitors of PI3Kα and mTOR. In this communication, we show that PI-103 cooperates with erlotinib in *PTEN*-mutant glioma, establishing a mechanistic rationale for the blockade of EGFR, PI3Kα, and mTOR in the treatment of *EGFR*-driven, *PTEN* mutant glioma.

Materials and Methods

Cell lines and reagents. LN229 and U87 cells transduced with *EGFRvIII* or *EGFR* as described (16) were grown in media containing 10% fetal bovine serum (FBS). Erlotinib tablets (Genentech) were ground to powder and dissolved in aqueous HCl, and aqueous phase was extracted with ethyl acetate. The combined organic extracts were dried over sodium sulfate and concentrated to yield pure erlotinib. Rapamycin was purchased from Cell Signaling; EGF was purchased from Roche Molecular Biochemicals; and PIK-90 and PI-103 were synthesized as described (9).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Cell proliferation assay and flow cytometry. For viability, 10^5 cells were seeded in 12-well plates in the presence of erlotinib, PI-103, PIK-90, rapamycin, erlotinib plus PIK-90, erlotinib plus rapamycin, erlotinib plus PIK-90, and rapamycin or erlotinib plus PI-103 for 72 h. Cell viability was determined using a WST-1 assay (Roche Molecular Biochemicals). Flow cytometry was as previously described (11).

Small interfering RNA transfection and retroviral infection. Synthetic control small interfering RNA (siRNA) and siRNA against PTEN were purchased from Santa Cruz Biotechnology and transfected using LipofectAMINE 2000 (Invitrogen) as previously described (16). pBabe puroL PTEN plasmid 10785 was purchased from Addgene. To generate the retrovirus, the packaging cell line 293T was cotransfected with plasmids expressing gag/pol and VSVg, using Effectene-transfected reagent (Qiagen). High-titer viruses were collected 48 h later and used to infect U87:*EGFR* cells as previously described (16).

Immunoblot analysis. Membranes were blotted with antisera to p-Akt (Ser^{473}), Akt, p-Erk (Ser$^{202/204}$), p-S6 ribosomal protein (Ser$^{235/236}$), and S6 ribosomal protein (Cell Signaling Technology), 4G10, β-tubulin (Upstate Biotechnology), Erk2, or EGFR (Santa Cruz Biotechnology). Immunoblotting and detection were as previously described (11).

Results

***PTEN* status and efficacy: erlotinib versus PI-103.** To clarify the role of *PTEN* as a determinant of response to inhibitors of EGFR/PI3K/mTOR signaling, we transduced EGFR into glioma cell lines LN229 and U87 and treated these with erlotinib or PI-103. In contrast to the *PTEN*^{mt} cell line U87:*EGFR*, LN229:*EGFR* cells (*PTEN*^{wt}) showed a prominent response to erlotinib (Fig. 1*A*). Flow-cytometric analysis showed G_0-G_1 arrest in LN229:*EGFR* cells (Fig. 1*B*). In comparison, U87:*EGFR* cells showed little response (Fig. 1*B*). These data are consistent with earlier studies that *PTEN* status represents an important determinant of response to EGFR inhibitors (6, 7).

Although mutation of PTEN would be expected to upregulate PI3K, and downstream signaling through Akt to mTOR [and subsequent feedback to PI3K (14–16)], we reasoned that *PTEN* status would not affect the ability of PI-103, a potent inhibitor of both PI3K and mTOR, to block the outputs of this signaling pathway and induce cell cycle arrest. Consistent with this model, PI-103 was equipotent against *PTEN*^{wt} and *PTEN*^{mt} cells, leading to arrest at G_0-G_1 (Fig. 1*A* and *B*). The response to this compound contrasted with the clear *PTEN* dependence observed using erlotinib and suggests that *PTEN* status is not a critical determinant of response to PI-103.

Erlotinib blocks mTOR in *PTEN*^{wt} cells. To explore downstream targets mediating the response of glioma cells to EGFR

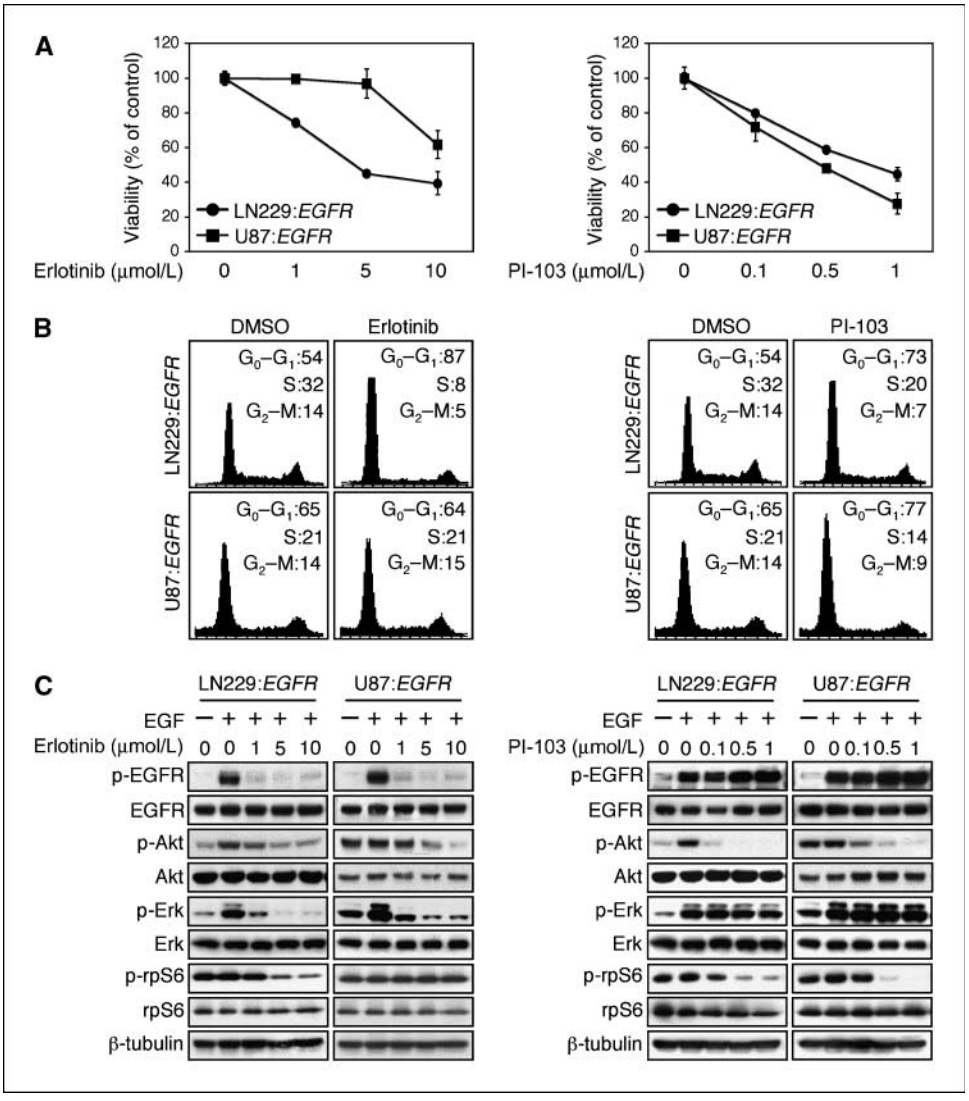

Figure 1. EGFR inhibitor erlotinib inhibits cell proliferation and induces G_0-G_1 arrest dependent on *PTEN* status. In contrast, antiproliferative effects of the dual PI3 kinase/mTOR inhibitor PI-103 were not dependent on *PTEN* status. LN229:*EGFR* (*PTEN*^{wt}) and U87:*EGFR* (*PTEN*^{mt}) cells were treated with erlotinib or PI-103. *A,* viability was determined using WST-1 assay after treatment with erlotinib or PI-103 in 12 wells for 72 h at dosages indicated. *Bars,* error between triplicate measurements. *B,* cells were treated with 5 μmol/L erlotinib or 0.5 μmol/L PI-103 for 24 h. Cells were trypsinized and prepared for flow-cytometric analysis of cell cycle distribution. Percentage of cells in G_0-G_1, S, and G_2-M phases of the cell cycle is indicated. *C,* the EGFR inhibitor erlotinib inhibits signaling through Akt and mTOR in a *PTEN*-dependent manner, and the dual PI3Kα/mTOR inhibitor PI-103 blocks both Akt and mTOR irrespective of *PTEN* status. LN229:*EGFR* (*PTEN*^{wt}) and U87:*EGFR* (*PTEN*^{mt}) cells were treated with erlotinib or PI-103 at dosages shown for 24 h. EGF (50 ng/mL) was added 15 min before harvest, and lysates were immunoblotted as indicated. Erlotinib therapy led to decreased Erk signaling in both cell lines, but impacted levels of p-Akt and the mTOR target p-rpS6 kinase only in cells wild-type for *PTEN*. Although U87:*EGFR* cells had higher baseline signaling through p-Akt, treatment with PI-103 led to dose-dependent blockade of both p-Akt and p-rpS6, without appreciably impacting levels of p-Erk.

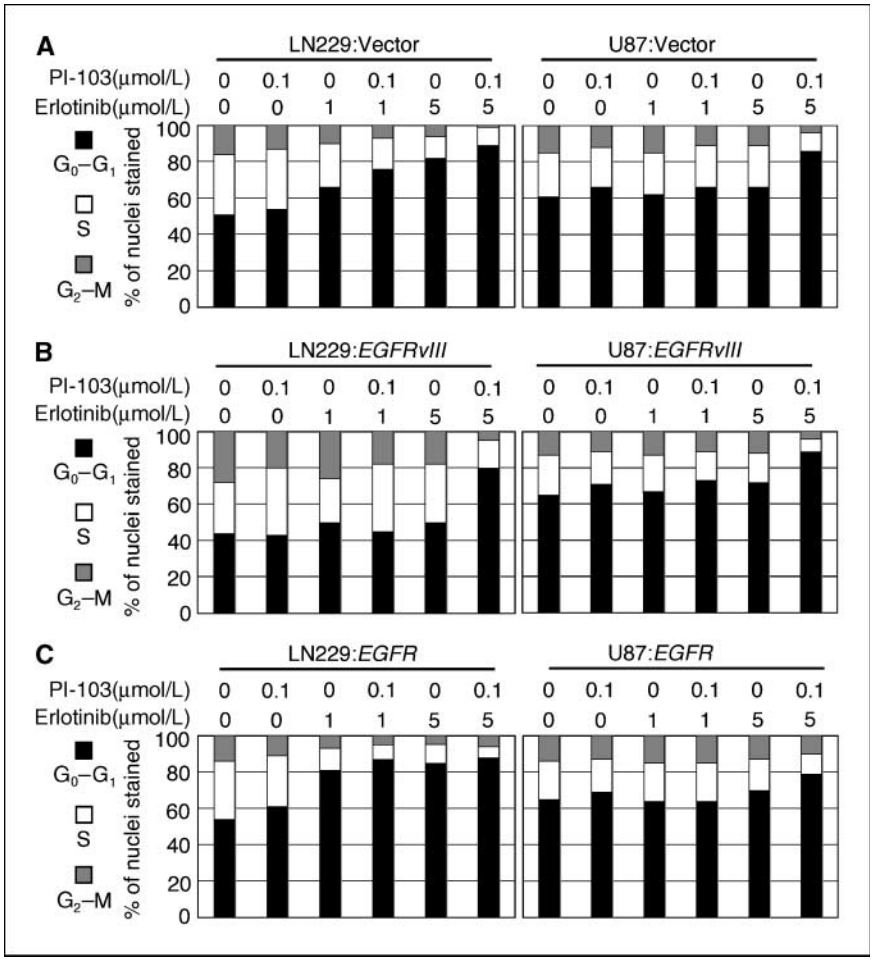

Figure 2. Inhibitors of EGFR cooperate with the dual PI3Kα/mTOR inhibitor PI-103 to arrest growth of *PTEN* mutant human glioma cells. LN229 (*PTEN*^{wt}) and U87 (*PTEN*^{mt}) cells transduced with vector (*A*), *EGFRvIII* (*B*), or wild-type *EGFR* (*C*) were treated with erlotinib, PI-103, or erlotinib plus PI-103 at indicated dosages. Flow cytometry analysis shows effects on cell cycle after treatment for 24 h. Percentage of cells in G_0-G_1, S, and G_2-M phases of the cell cycle is indicated.

blockade, we did immunoblotting of *PTEN*^{wt} and *PTEN*^{mt} cells in response to erlotinib using phosphospecific antibodies. Treatment of cells with EGF led to equivalent responses in mitogen-activated protein (MAP) kinase signaling irrespective of *PTEN* status, as indicated by levels of p-Erk (Fig. 1*C*). Inhibition of EGFR impacted p-Erk similarly in both cell lines, consistent with pathways linking EGFR to MAP kinase signaling that were not impacted by *PTEN* status (Fig. 1*C*). In contrast, although treatment with EGF led to activation of p-Akt in *PTEN*^{wt} cells, EGF showed little impact on p-Akt in *PTEN*^{mt} cells (Fig. 1*C*). *PTEN*^{mt} cells had high levels of p-Akt at baseline, consistent with the activation of PI3K through the loss of *PTEN*. These high levels of p-Akt were only modestly affected by the treatment of *PTEN*^{mt} cells with erlotinib (Fig. 1*C*) and stood in contrast to comparable experiments treating *PTEN*^{wt} cells with this compound (Fig. 1*C*), again supporting a model in which the loss of *PTEN* effectively uncouples the activation of PI3K/Akt from upstream signaling through *EGFR*.

To provide mechanistic insights with regard to molecular alterations occurring as a result of *PTEN* mutation in combination with EGFR overexpression, we did similar experiments in LN229 cells and U87 cells expressing endogenous levels of EGFR or expressing the constitutively active tumor-derived *EGFRvIII* allele. LN229 (*PTEN*^{wt}) cells expressing endogenous levels of EGFR again responded to erlotinib, whereas U87 (*PTEN*^{mt}), cells were resistant (Supplemental Fig. S1*A*). In contrast, cells transduced with *EGFRvIII* were moderately resistant to erlotinib even in the setting of wild-type *PTEN* (Supplemental Fig. S1*A*). EGFR blockade only minimally impacted phosphorylation of both EGFRvIII and Akt in LN229:*EGFRvIII* and U87:*EGFRvIII* cells (Supplemental Fig. S1B), consistent with observations that the *EGFRvIII* allele is resistant to tyrosine kinase inhibition (17).

To evaluate the response of mTOR signaling, we analyzed the mTOR target ribosomal protein S6 kinase (rpS6). At baseline, levels of p-rpS6 were prominent in both lines, apparently unaffected by *PTEN* status. In contrast, *PTEN* status was important in determining whether the blockade of EGFR affected p-rpS6. In *PTEN*^{wt} cells, treatment with erlotinib led to decreased p-rpS6, which changed in parallel with p-Akt (Fig. 1*C*). Importantly, treatment of *PTEN*^{mt} cells with erlotinib did not impact p-rpS6, even at doses sufficiently high to block signaling through p-Akt (Fig. 1*C*). These data show that *PTEN* links *EGFR* to mTOR, that inhibition of EGFR can block signaling through mTOR in *PTEN*^{wt} but not in *PTEN*^{mt} cells, and suggest that blockade of mTOR correlates with the efficacy of EGFR inhibitors.

We next asked whether *PTEN* status was a determinant of the biochemical response to PI-103. Both *PTEN*^{wt} and *PTEN*^{mt} cells showed qualitatively similar blockade of p-Akt and p-rpS6 in response to PI-103 (Fig. 1*C*), whereas levels of p-Erk were only minimally affected. These experiments are consistent with results in Fig. 1*A* and *B*, demonstrating that PI-103 was equipotent in blocking proliferation in both *PTEN*^{wt} and *PTEN*^{mt} cells.

Erlotinib cooperates with PI-103 to arrest cells. Results in Fig. 1 argue that mutation at *PTEN* uncouples EGFR from downstream signaling through PI3K and mTOR, suggesting that

the blockade of PI3K and/or mTOR could enhance the efficacy of EGFR inhibition in *PTEN*^{mt} glioma. To address the efficacy of this combination with regard to molecular alterations occurring as a result of *PTEN* mutation in combination with EGFR overexpression, we treated cells with erlotinib in combination with PI-103 in the settings of endogenous levels of EGFR and in cells transduced with either the tumor-derived *EGFRvIII* allele or with wild-type *EGFR* in both LN229 *PTEN*^{wt} and U87 *PTEN*^{mt} cells (Fig. 2). As expected, erlotinib led to G_0-G_1 arrest in *PTEN*^{wt} LN229:vector cells or LN229:*EGFR* cells and, with a 5-μmol/L dose, was minimally augmented by PI-103 (Fig. 2*A* and *C*). In contrast, erlotinib with a dose of 5 μmol/L had little impact as monotherapy in *PTEN*^{mt} cells and *PTEN*^{wt} LN229:*EGFRvIII* cells. However, erlotinib did show efficacy when combined with low-dose (100 nmol/L) PI-103 in this setting, with combination therapy effectively promoting arrest at G_0-G_1 (Fig. 2).

Immunoblotting experiments further reinforced results in Fig. 2, demonstrating that *PTEN* status correlated with the ability of erlotinib monotherapy to impact signaling through mTOR, regardless of EGFR levels, and was consistent in cells transduced

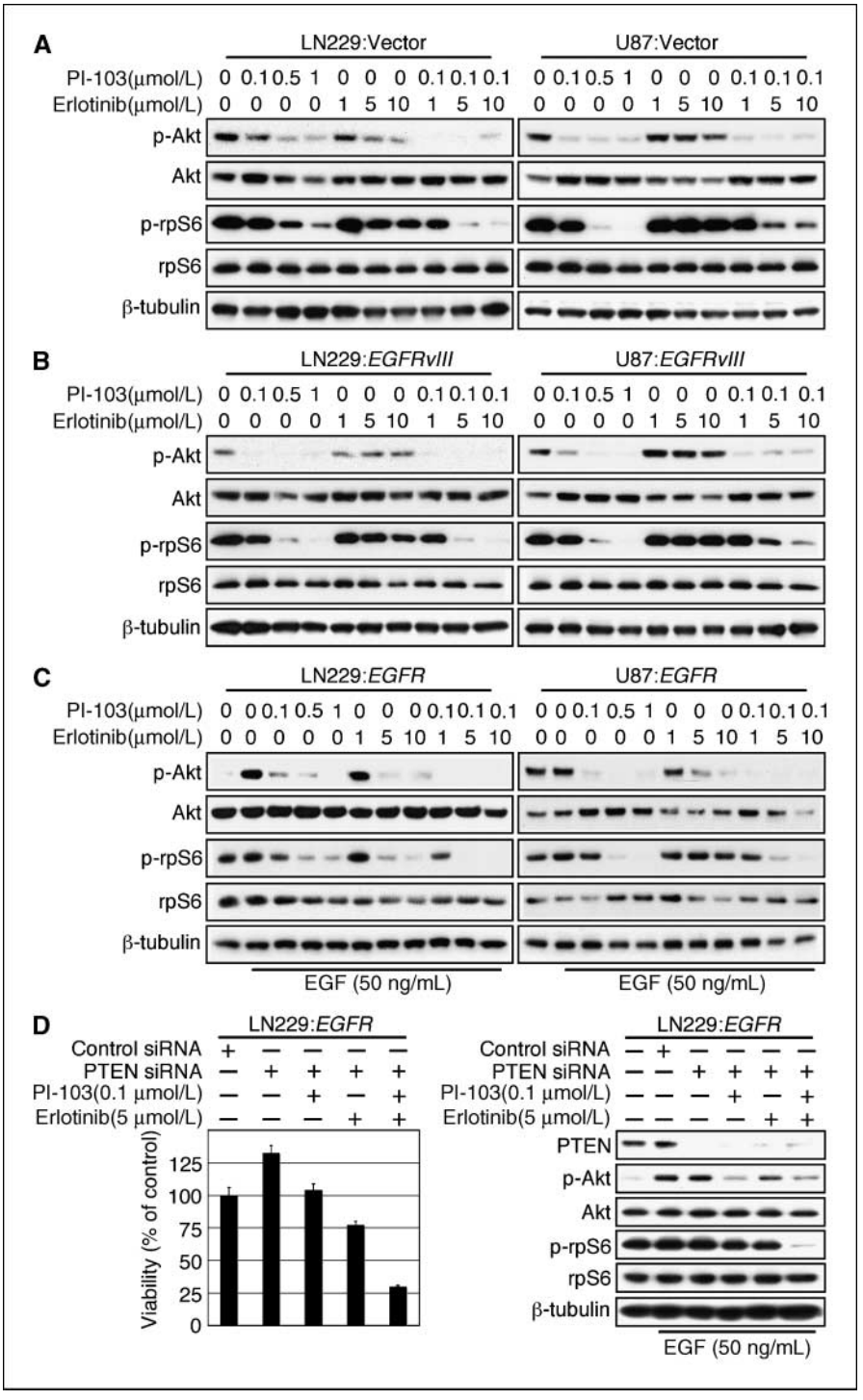

Figure 3. *PTEN* status correlates with the ability of erlotinib monotherapy to impact signaling through mTOR. *A–C,* cells were treated as in Fig. 2. EGF (50 ng/mL) was added in (*C*) 15 min before harvest. Cell lysates were subjected to immunoblot analysis with antisera indicated. *D,* LN229:*EGFR* (*PTEN*^{wt}) cells were transfected with control siRNA or siRNA against PTEN for 24 h and then treated with inhibitors indicated for 24 h. Viability was measured by WST-1 assay, and levels of signaling molecules were measured by Western blot. In comparison with Fig. 1*A*, erlotinib treatment of *PTEN* knockdown cells led to increased viability (*left, fourth column*), correlating with increased levels of p-Akt and p-rps6 (*right, fifth lane*).

with wild-type *EGFR* and *EGFRvIII* (Fig. 3*A–C*). As monotherapy, erlotinib could block signaling through mTOR most effectively in *PTEN*^{wt} cells. Addition of PI-103 to erlotinib in these cells further blocked signaling through mTOR. In contrast, mutation at *PTEN* was an important and negative determinant of erlotinib's ability to impact the activation of mTOR. Whereas treatment of *PTEN*^{mt} cells with erlotinib did not impact the mTOR target p-rpS6, addition of PI-103 to erlotinib in these cells led to blockade of p-rpS6 (Fig. 3) and effected arrest at G_0-G_1 (Fig. 2). These observations again suggest a model in which *PTEN* status correlates with the ability of EGFR inhibitors to impact signaling through mTOR and supports combining PI-103 with erlotinib in *EGFR*-driven *PTEN*^{mt} tumors.

To verify the importance of *PTEN* in mediating relative sensitivity and resistance to erlotinib, we used siRNA to knock down *PTEN* in *PTEN*^{wt} cells (Fig. 3*D*). Knockdown of *PTEN* in LN229:*EGFR* cells led to increased viability in the absence of treatment and mediated relative resistance to erlotinib [45.1% viability in LN229:EGFR cells treated with 5 μmol/L erlotinib (Fig. 1*A*), compared with 77% viability with *PTEN* knockdown (Fig. 3*D*)]. Immunoblotting revealed that erlotinib loses potency against p-Akt and p-rpS6 as a result of *PTEN* knockdown (Fig. 3*D*). We also transduced wild-type *PTEN* into *PTEN*^{mt} U87:*EGFR* cells (Supplementary Fig. S2). This manipulation led to decreased viability in the absence of treatment and increased sensitivity to erlotinib [84.2% viability in U87:*EGFR* cells treated with 5 μmol/L erlotinib (Supplementary Fig. S3*A*), compared with 55.3% viability with transduced wild-type *PTEN* (Supplementary Fig. S2*A*)], associated with more efficient blockade of p-Akt. Interestingly, however, levels of p-rpS6 were unchanged (Supplementary Fig. S2*B*), an observation also reported by others (18).

Blockade of EGFR, PI3Kα, and mTOR in glioma. Having shown that that PI-103 can augment the response to erlotinib in *PTEN*^{mt} cells, we next asked whether all three targets of these agents (EGFR, PI3Kα, and mTOR) were critical to maximal proliferative blockade. We treated *PTEN*^{mt} U87:*EGFR* cells with erlotinib in combination with the pure PI3Kα inhibitor PIK-90, the mTOR inhibitor rapamycin, combination therapy using both PIK-90 and rapamycin, or the dual PI3Kα/mTOR inhibitor PI-103 (Supplementary Fig. S3). We also treated *PTEN*^{mt} U87:*EGFRvIII* cells and did a comparable but more extensive analysis analyzing each inhibitor individually and assessing the impact of paired inhibitors, comparing erlotinib plus PI-103 to triple therapy using erlotinib, rapamycin, and PIK-90 (Fig. 4). Measures of overall viability and of proliferation were consistent, showing that blockade of mTOR cooperated with inhibition of EGFR, and that further blockade of PI3Kα led to maximal proliferation block (Fig. 4 compare *column 7* to *8* or *9*; Supplementary Fig. S3*A*, compare *columns 7* and *9, P* < 0.0001 Student's *t* test). Terminal nucleotidyl transferase–mediated nick end labeling and sub-G_1 fractions showed no significant difference in apoptosis among these therapies (data not shown).

Immunoblotting experiments (Fig. 4, Supplementary Fig. S3*B*) were aligned with flow cytometry. Cooperative inhibition of EGFR and of mTOR led to decreased levels of both p-EGFR and p-rpS6; however, rapamycin actually increased levels of p-Akt (Fig. 4, Supplementary Fig. S3*B*). Whereas erlotinib could efficiently block p-EGFR (Fig. 4, Supplementary Fig. S3*B*), combining erlotinib with either PIK-90 or rapamycin blocked either p-Akt or p-rpS6 (Fig. 4, Supplementary Fig. S3*B*). Further inhibition of PI3Kα was required to simultaneously block p-Akt and p-rpS6 (Fig. 4, Supplementary Fig. S3*B*). These results show that blockade of EGFR and of mTOR cooperate in the treatment of *EGFR*-driven, *PTEN*^{mt} glioma, and that further efficacy can be achieved through concomitant blockade of PI3Kα.

Discussion

The malignant gliomas show intrinsic resistance to most medical therapies, contributing to their poor prognosis (1). The association of *EGFR* amplification with high-grade glioblastoma multiforme tumors therefore led to early optimism that EGFR inhibition would be beneficial. This initial optimism was mitigated by observations that only a subset of patients with *EGFR*-amplified glioma actually responded to the blockade of EGFR (19). The failure of this approach in the majority of patients could stem from inefficient blockade of EGFR or from the inability to reverse signaling abnormalities associated with *EGFR* amplification, even in the setting of adequate blockade. Loss of *PTEN* contributes to this failure because loss of *PTEN* blocks the ability of EGFR inhibitors to impact downstream signaling through PI3K and mTOR.

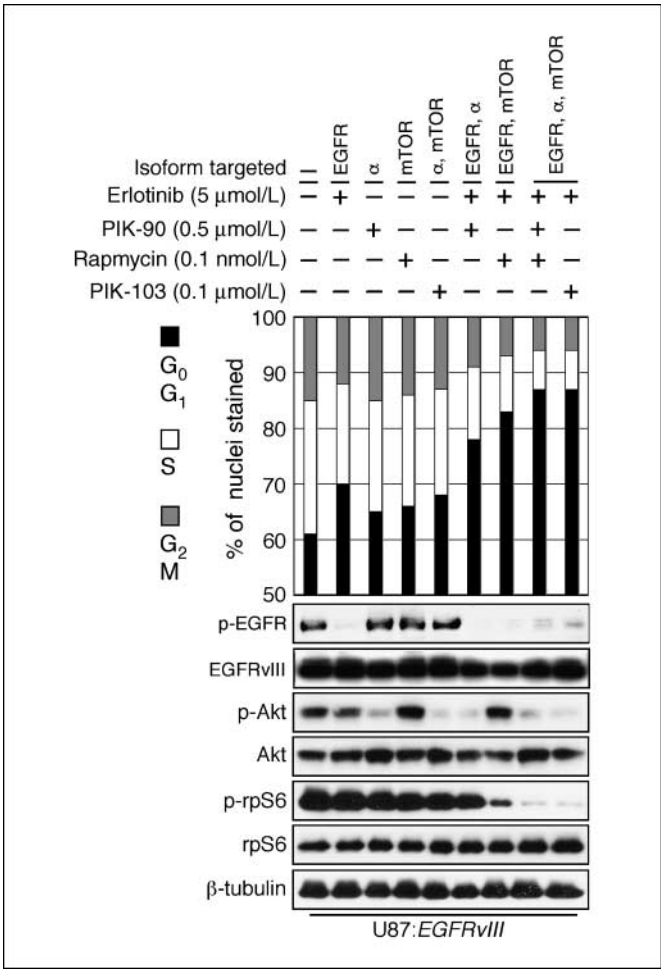

Figure 4. Cooperative inhibition of EGFR, p110α, and mTOR are critical to arrest growth of *PTEN*^{mt} human glioma cells transduced with *EGFRvIII*. U87:*EGFRvIII* (*PTEN*^{mt}) cells were treated with erlotinib; PIK-90; rapamycin; PI-103; erlotinib plus PIK-90; erlotinib plus rapamycin; erlotinib plus PIK-90 and erlotinib triple therapy; or erlotinib plus PI-103 at dosages indicated. Flow cytometry analysis shows effects on cell cycle after treatment for 24 h. Percentage of cells in G_0-G_1, S, and G_2-M phases of the cell cycle is indicated (*top*). Cell lysates were subjected to immunoblot analysis with antisera indicated (*bottom*).

In this communication, we present a preclinical approach aimed at reversing signaling abnormalities associated with *EGFR* amplification, offering a mechanistic rationale to combine inhibitors of EGFR and of mTOR to effect proliferation blockade in patients with *EGFR*-amplified, *PTEN*^{mt} glioma. We showed the efficacy for inhibitors of EGFR as monotherapy in glioma cells wild-type for *PTEN*, and that the antiproliferative effect of EGFR inhibitors correlated with the ability of these agents to impact levels of mTOR.

In contrast to *PTEN*^{wt} cells, erlotinib treatment of *PTEN*^{mt} cells did not appreciably impact proliferation and specifically did not impact mTOR, even when inhibitors of EGFR were used at doses sufficiently high to block p-Akt (Fig. 1*C*). Although erlotinib had little measurable activity as monotherapy in *PTEN*^{mt} cells, erlotinib clearly augmented the efficacy of PI-103 as measured both by blockade of mTOR and of proliferation (Fig. 4). Intriguingly, the ability of PI-103 and erlotinib to impact mTOR again was observed in a setting where combination therapy did not appreciably alter levels of p-Akt in comparison with PI-103 alone (Fig. 4). The dissociation of Akt from mTOR in *PTEN*^{mt} glioma has also been observed by others (18) and suggests the presence of Akt-independent regulators of mTOR.

The failure of inhibitors of EGFR to impact mTOR signaling in *PTEN*^{mt} glioma also provides a rationale to combine inhibitors of EGFR and mTOR. While targeting both kinases simultaneously led to decreased proliferation in comparison with targeting either EGFR or mTOR alone (Fig. 4), blockade of mTOR by rapamycin actually led to increased levels of p-Akt (Fig. 4), a phenomenon previously described by others (14, 15).

In response to the failure of EGFR inhibitors to block PI3K, Akt, or mTOR in *PTEN*^{mt} glioma, and because mTOR inhibitors actually activate the PI3K/Akt axis, we tested inhibitors of EGFR and of mTOR in combination with inhibitors of PI3Kα. The combinatorial inhibition of three targets effectively blocked signaling and was more effective than any two targeted therapies in combination as measured both biochemically and through flow-cytometric analyses (Fig. 4). Even using approaches that blocked EGFR, PI3Kα, and mTOR in combination, and in the setting of efficient inhibition of Akt, an important mediator of antiapoptotic signaling (20), we failed to observe any appreciable apoptosis in any glioma cell lines tested. Thus, whereas the translation of our findings to patients awaits clinical development of isoform-specific inhibitors of PI3K, the ability to induce cytotoxic rather than cytostatic responses in the malignant gliomas represents a more formidable challenge and one that may be critical to the long-term efficacy of these approaches in patients.

Acknowledgments

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