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A dual PI3K α/mTOR inhibitor cooperates with blockade of EGFR in *PTEN*-mutant glioma

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Abstract

We have shown previously that blockade of EGFR cooperates with a pan-selective inhibitor of 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, without further impact by PI-103. 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 demonstrate 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.

Keywords

Glioma; EGFR; PTEN; mTOR; PI-103

Introduction

Both amplification of EGFR and activation of phosphatidylinositol-3 kinase (PI3K) feature prominently in glioma (Persson et al., 2007). Activation of PI3K may occur as a consequence of *EGFR* amplification, and in such cases should respond to inhibitors of EGFR. PI3K may also be activated independently of EGFR, through gain-of-function mutations in PI3K itself or from inactivation of the lipid phosphatase *PTEN,* a negative regulator of PI3K (Vogt et al.,

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2006; Zhao and Roberts, 2006). Because activation of PI3K is uncoupled from upstream amplification of *EGFR* in *PTEN*-mutant glioma, we previously tested inhibitors of PI3K and of EGFR in combination, demonstrating efficacy for this approach in *EGFR*-driven, *PTEN*-mutant glioma cell lines and xenografts (Fan et al., 2003). The clinical relevance of this work was recently corroborated by observations in patients treated with inhibitors of EGFR. In two retrospective analyses, tumors in which activation of PI3K was coupled to *EGFR* amplification responded to inhibition of EGFR (Haas-Kogan et al., 2005; Mellinghoff et al., 2005). In contrast, tumors in which PI3K was activated independently of *EGFR* responded poorly to EGFR inhibition. Collectively, these reports suggest that in tumors with *EGFR* amplification and *PTEN* inactivation (comprising half of *EGFR*-amplified glioma), combining inhibitors of EGFR and inhibitors of PI3K represents a promising therapy.

What are the hurdles preventing translation of this approach to patients? The eight mammalian PI3-kinases are grouped within three classes (Engelman et al., 2006). Although pan-selective inhibitors of PI3Ks have been critical to our current understanding, these compounds indiscriminately inhibit all known PI3Ks and are toxic in patients (Workman et al., 2006). To delineate the role of individual PI3Ks, we recently synthesized a series of isoform-selective inhibitors of PI3Ks, defined the structural basis for their specificity, and systematically enumerated their biochemical targets (Knight et al., 2006). These agents, in conjunction with similar compounds described by others (Camps et al., 2005; Hayakawa et al., 2007; Hayakawa et al., 2006; Jackson et al., 2005; Knight et al., 2004; Sadhu et al., 2003) collectively represent an important arsenal of tools for analysis of signaling through PI3K. 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 (Fan et al., 2006).

We noted particular efficacy in combining inhibitors of PI3Kα with inhibitors of mTOR, a serine-threonine kinase that is part of a protein complex critical for cell growth (Corradetti and Guan, 2006; Sabatini, 2006). This result was at first somewhat surprising, as mTOR is activated as a consequence of signaling through PI3K. PI3Ks phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP_2), generating phosphatidylinositol-4,5-trisphosphate (PIP_3), which in turn activates Akt, a PH-domain containing serine-threonine kinase that signals through downstream effecters including mTOR, to suppress apoptosis, promote cell growth, and drive proliferation (Shaw and Cantley, 2006). Importantly, inhibitors of mTORC1 actually activate signaling through PI3K (Fan et al., 2006; Shi et al., 2005; Sun et al., 2005). 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 PI3Kα and mTOR shuts down mTOR, and also abrogates the activation of PI3K observed using mTOR inhibitors as monotherapy. 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 was highly effective against glioma xenografts (Fan et al., 2006).

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

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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 the glioma cell lines LN229 and U87, and treated these with erlotinib, or with PI-103. In contrast to the $PTEN^{mt}$ cell line U87:*EGFR*, LN229:*EGFR* cells ($PTEN^{wt}$) showed a prominent response to erlotinib (Fig 1A-B). Flow cytometric analysis demonstrated G_0G_1 arrest in LN229 cells (Fig 1C). In comparison, U87:*EGFR* cells showed a more modest response (Fig 1C). These data are consistent with results by others that *PTEN* status represents an important determinant of response to EGFR inhibitors (Haas-Kogan et al., 2005;Mellinghoff et al., 2005).

Since mutation at *PTEN* should not interfere with pathways coupling PI3K to mTOR, we reasoned that *PTEN* status might be less important for the dual inhibitor PI-103. Consistent with this model, PI-103 was equipotent against $PTEN^{wt}$ and $PTEN^{mt}$ cells, leading to arrest at G_0G_1 (Fig 1 D-F). 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 the dual PI3K/mTOR inhibitor PI-103.

Erlotinib blocks mTOR in $PTEN^{wt}$ cells

To explore downstream targets mediating the response of glioma cells to EGFR blockade, we performed immunoblotting of $PTEN^{wt}$ and $PTEN^{mt}$ cells in response to erlotinib using phospho-specific antibodies. Treatment of cells with EGF led to equivalent responses in MAP-kinase signaling irrespective of *PTEN* status, as indicated by levels of p-Erk (Fig 2). Inhibition of EGFR impacted levels of p-Erk similarly in both cell lines, consistent with pathways linking EGFR to MAP kinase signaling that were not impacted by *PTEN* status (Fig 2A). In contrast, although treatment with EGF led to activation of p-Akt in $PTEN^{wt}$ cells (which showed increased levels of p-Akt), EGF showed little impact on p-Akt in $PTEN^{mt}$ cells. $PTEN^{mt}$ cells had high levels of p-Akt at baseline, consistent with activation of PI3K through loss of *PTEN*, a negative regulator of PI3K signaling. These high levels of p-Akt were only modestly affected by treatment of $PTEN^{mt}$ cells with erlotinib, and stood in contrast to comparable experiments treating $PTEN^{wt}$ cells with this compound (Fig 2A), again supporting a model in which loss of *PTEN* effectively uncouples activation of PI3K/Akt from upstream signaling through *EGFR.*

To address 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 cell lines, apparently unaffected by *PTEN* status, and similarly (in these cells, grown in 10% FBS) unaffected by treatment with EGF. In contrast, *PTEN* status was important in determining whether blockade of EGFR affected levels of p-rpS6. In $PTEN^{wt}$ cells, treatment with erlotinib led to decreased levels of p-rpS6, which changed in parallel with p-Akt (Fig 2A). Importantly, treatment of $PTEN^{mt}$ cells with erlotinib did not impact levels of p-rpS6, even at doses sufficiently high to block signaling through p-Akt. These data demonstrate 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 efficacy of EGFR inhibitors.

We next asked whether *PTEN* status was a determinant of the biochemical response to the dual inhibitor PI-103. Both $PTEN^{wt}$ and $PTEN^{mt}$ cells showed significant blockade of p-Akt in response to PI-103 (Fig 2B), whereas levels of p-Erk were only minimally affected. While base-line levels of p-rpS6 showed minor differences comparing $PTEN^{wt}$ and $PTEN^{mt}$ cells, the response to PI-103 was qualitatively similar. These experiments are consistent with results

in Fig 1, demonstrating that PI-103 was equipotent in blocking proliferation in both *PTEN*^{wt} and *PTEN*^{mt} cells, suggesting that *PTEN* status did not correlate with response to this agent.

Erlotinib cooperates with PI-103 to arrest cells

Collectively, data in Figs 1-2 argues that mutation at *PTEN* uncouples EGFR from downstream signaling through PI3K and mTOR, suggesting that blockade of PI3K and/or mTOR could enhance the efficacy of EGFR inhibition in *PTEN*^{mt} glioma. To address the efficacy of this combination, we treated cells with erlotinib in combination with PI-103 (Fig 3). As expected, erlotinib led to G_0G_1 arrest in *PTEN*^{wt} cells, and was minimally augmented by PI-103. In contrast, erlotinib had little impact as monotherapy in *PTEN*^{mt} cells. Erlotinib did show efficacy when combined with low-dose (100 nM) PI-103 in this setting however, with combination therapy effectively promoting arrest at G_0G_1 (Fig 3A).

Immunoblotting experiments further reinforced results in Fig 2A, demonstrating that *PTEN* status correlated with the ability of erlotinib monotherapy to impact signaling through mTOR (Fig 3B). As monotherapy, erlotinib could block signaling through mTOR most effectively in *PTEN*^{wt} cells. Addition of PI-103 to erlotinib in these cells only minimally altered the efficiency through which erlotinib monotherapy blocked signaling through mTOR. In contrast, mutation at *PTEN* was an important and negative determinant of erlotinib's ability to impact activation of mTOR. Whereas treatment of *PTEN*^{mt} cells with erlotinib minimally affected levels of the mTOR target p-rpS6, addition of PI-103 to erlotinib in these cells led to effective blockade of p-rpS6 (Fig 3B). These observations again support 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.

Blockade of EGFR, PI3Kα, and mTOR in glioma

Having demonstrated 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 achieve maximal proliferative blockade. We treated *PTEN*^{mt} 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. Measures of overall viability and of proliferation were consistent in showing that blockade of mTOR cooperated with inhibition of EGFR, and that further blockade of PI3Kα led to maximal proliferation block (Fig 4A, compare columns 7 and 9, $p<0.0001$-Student's t-test). TUNEL and subG1 fractions demonstrated no significant difference in apoptosis among these therapies (not shown). Immunoblotting experiments (Fig 4B) were aligned with results in Fig 4A. While cooperative inhibition of EGFR and of mTOR led to decreased levels of both p-EGFR and p-rpS6, treatment with rapamycin actually increased levels of p-Akt. Further inhibition of PI3Kα was required to efficiently block p-Akt in the setting of efficient blockade of both p-EGFR and p-rps6. These results demonstrate 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 the poor prognosis associated with these tumors (Persson et al., 2007). The association of *EGFR* amplification with high-grade glioblastoma multiforme tumors therefore led to early optimism that EGFR inhibition would be beneficial in glioma. This initial optimism was mitigated however, by the realization that only a subset of patients with *EGFR*-amplified glioma actually responded to blockade of EGFR (Nicholas et al., 2006). The failure of this approach in the majority of patients with *EGFR*-amplified glioma could stem from inefficient blockade of the

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receptor or from inability to reverse signaling abnormalities associated with *EGFR* amplification, even in the setting of adequate blockade of p-EGFR. Loss of *PTEN* is a likely contributor to this failure, as loss of *PTEN* effectively blocks the ability of EGFR inhibitors to impact downstream signaling through PI3K and ultimately through mTOR.

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 demonstrated 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 2). 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 (Wang et al., 2006), 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 4B, lane 5). The activation of p-Akt by rapamycin and its analogues has been described previously in primary human tumors (O'Reilly et al., 2006;Shi et al., 2005;Sun et al., 2005). Addition of an mTOR inhibitor effectively blocks mTOR, but at the cost of activating other targets of PI3K and Akt.

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 (Fig 3), and was more effective than any two targeted therapies in combination as measured both biochemically and through flow cytometric analyses (Fig 4). It is intriguing in this regard that even using approaches that blocked EGFR, PI3Kα, and mTOR in combination, and in the setting of efficient inhibition of Akt—an important mediator of anti-apoptotic signaling (Luo et al., 2003)—we failed to observe any appreciable apoptosis in any glioma cell lines tested (Fig 4 and data not shown). Thus, while the ability to translate our findings to patients awaits the development of isoform-specific inhibitors of PI3K that are well tolerated clinically, the ability to develop targeted agents that 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.

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METHODS

Growth inhibition, cell staining, and flow cytometry

LN229 and U87 cells transduced with EGFR as described (Fan and Weiss, 2005), were grown in media containing 10% FBS. *PTEN*^{wt} and *PTEN*^{mt} cells transduced with ΔEGFR were also examined in experiments similar to those in Figs 1-4, with comparable results (not shown). Erlotinib tablets (Genentech) were ground to powder, dissolved in aqueous HCl and aqueous phase extracted with ethyl acetate. The combined organic extracts were dried over sodium sulphate and concentrated to yield pure erlotinib. PIK-90 and PI-103 were synthesized as described (Knight et al., 2006). For viabilty, 10^5 cells were seeded in 12-well plates in the presence of erlotinib, PI-103, PIK-90, rapamycin (Cell Signaling), erlotinib plus PIK-90, erlotinib plus rapamycin, PIK-90 plus rapamycin or erlotinib plus PI-103 for 3 d. Cell viability was determined using a WST-1 assay (Roche). For crystal-violet staining, 10^5 cells were seeded in 12 well plates +/− PI-103, erlotinib, or PI-103 plus erlotinib. After 3d, cells were washed with water and stained with crystal-violet for 5 min. Flow cytometry was as previously described (Fan et al., 2006).

Immunoblotting

Membranes were blotted with antisera to p-Akt (Ser473), Akt, p-Erk (Ser202/204), p-S6 ribosomal protein (Ser235/236), and S6 ribsomal protein (Cell Signaling), 4G10, β-tubulin (Upstate Biotechnology), Erk2, or EGFR (Santa Cruz). Immunoblotting and detection were as previously described (Fan et al., 2002).

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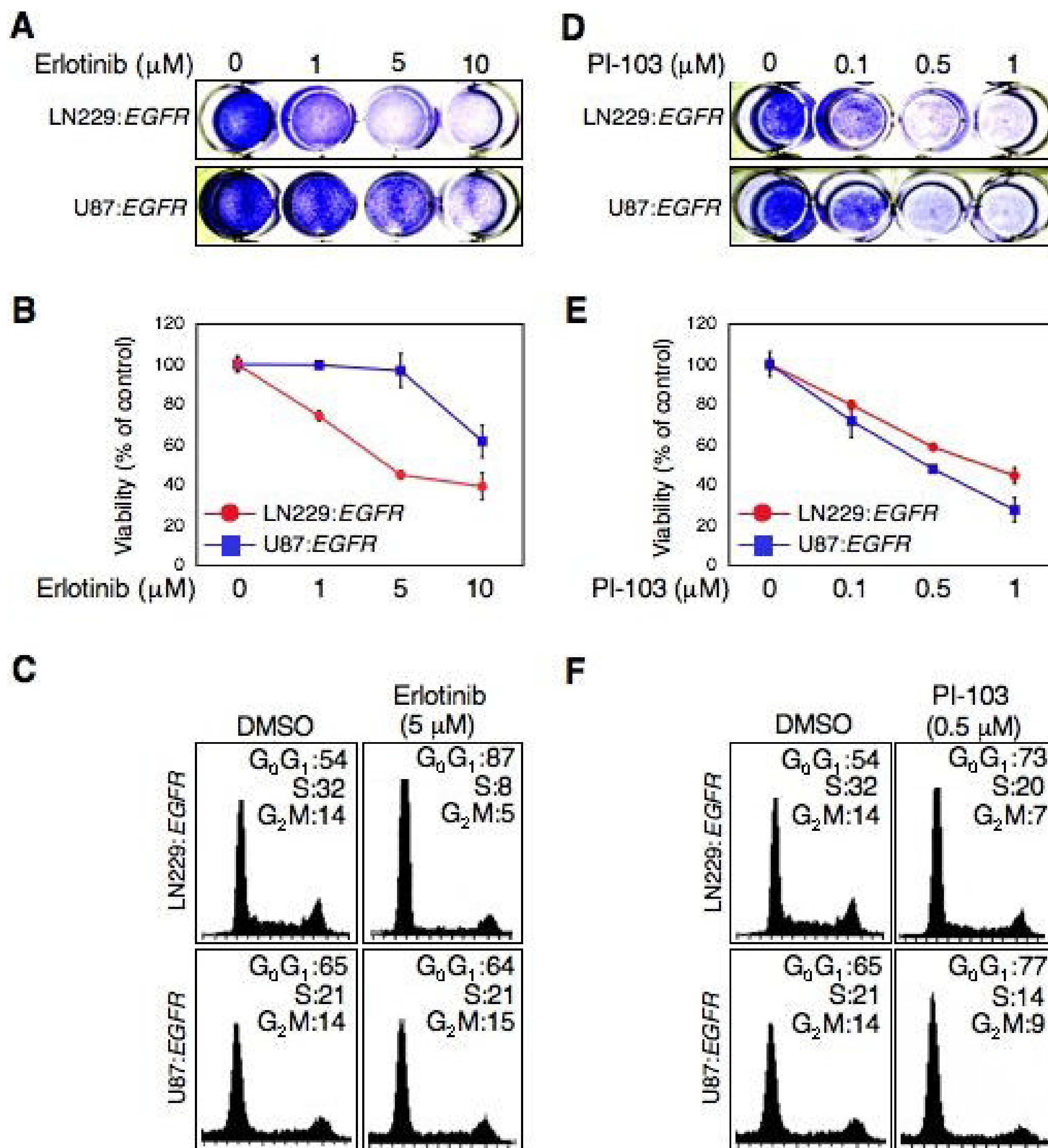

Fig 1.

EGFR inhibitor erlotinib inhibits cell proliferation and induces G_0G_1 arrest dependent on *PTEN* status. In contrast, anti-proliferative 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 and D)** Cell proliferation was visualized by crystal-violet staining of cells after treatment with erlotnib or PI-103 for 72h at dosages indicated. (**B and E)** Cell growth was determined using WST-1 assay after treatment with erlotinib or PI-103 for 72h at dosages indicated. Error between triplicate measurements for each value shown. (**C and F)** Cells were treated with erlotinib 5 μM or PI-103 0.5 μM for

24 hr. Cells were trypsinized and prepared for flow cytometric analysis of cell cycle distribution. Percentage of cells in G_0G_1, S, and G_2M phases of the cell cycle is indicated.

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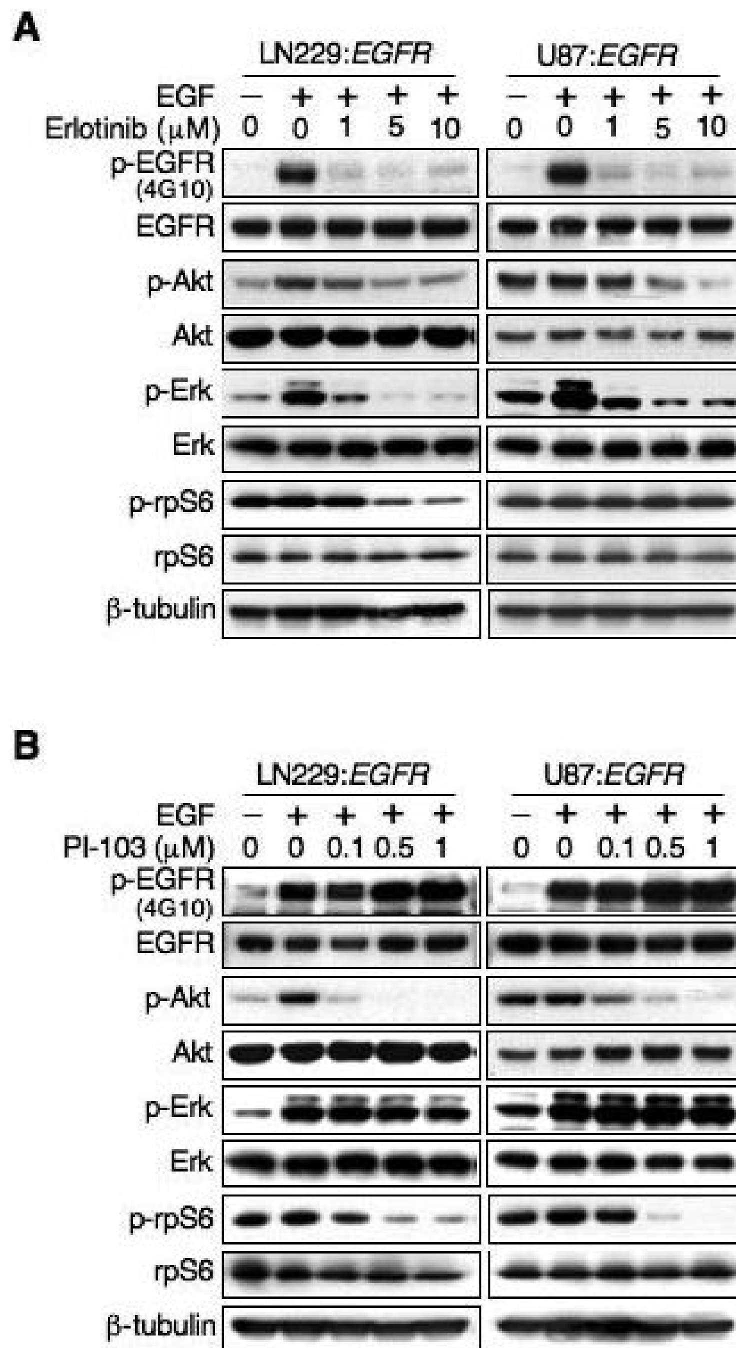

Fig 2.

Impact of PTEN status on biochemical response to Erlotinib or PI-103 treatment. (**A)** The EGFR inhibitor erlotinib inhibits signaling through Akt and mTOR in a *PTEN* dependent manner. LN229:*EGFR* (*PTEN*^{wt}) and U87:*EGFR* (*PTEN*^{mt}) cells were treated with erlotinib or PI-103 at dosages shown for 24 hr. EGF was added 15 min prior to harvest, and lysates 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*. (**B**) The dual PI3Kα/mTOR inhibitor PI-103 blocks both Akt and mTOR irrespective of *PTEN* status. Experimental conditions were identical to (**A**)**.** Although U87*:EGFR* cells had

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higher base-line 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.

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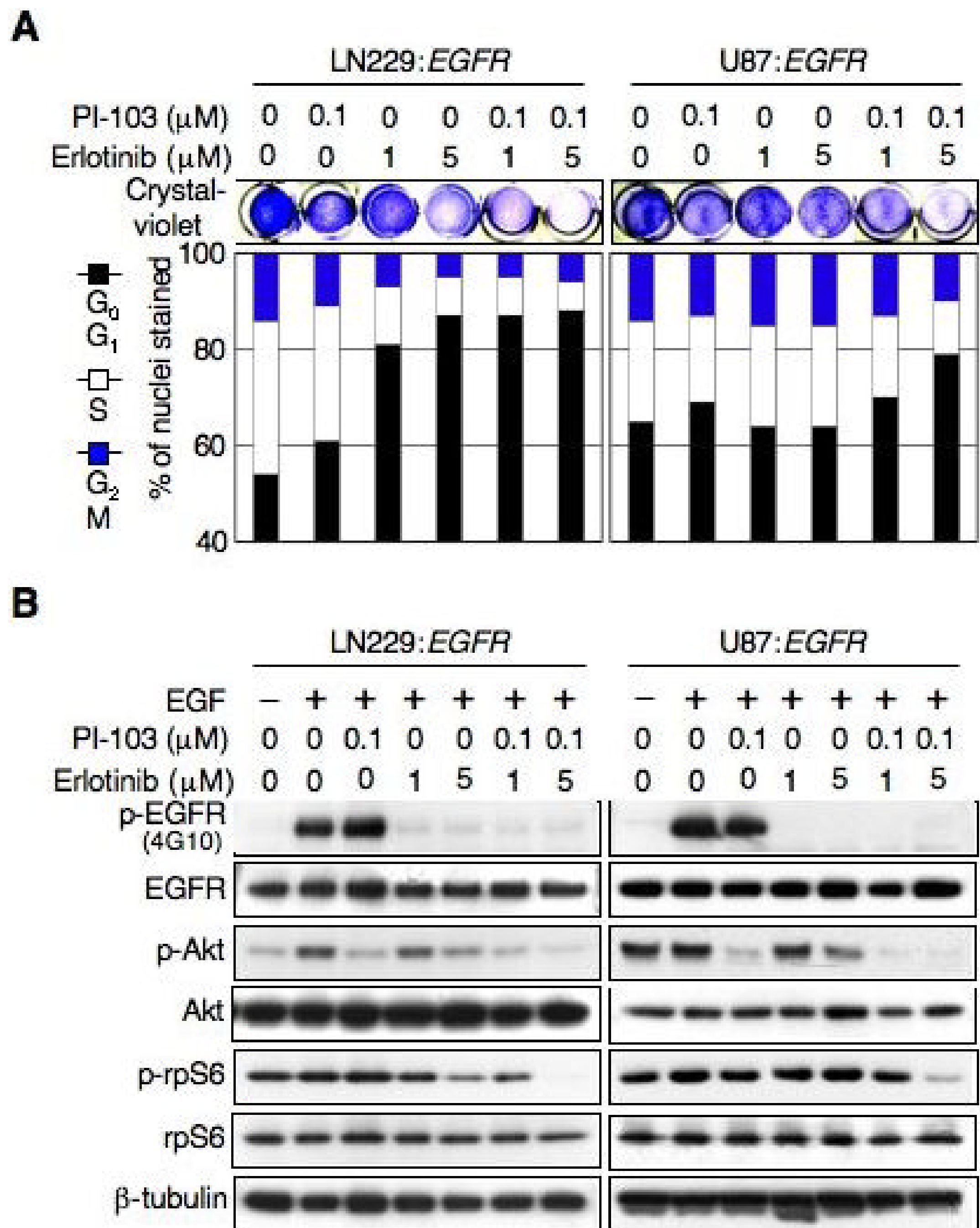

Fig 3.

Inhibitors of EGFR cooperate with the dual PI3Kα/mTOR inhibitor PI-103 to arrest growth of *PTEN*-mutant human glioma cells. LN229:*EGFR* (*PTEN*^{wt}) and U87:*EGFR* (*PTEN*^{mt}) cells were treated with erlotinib, PI-103, or erlotinib plus PI-103 at indicated dosages. (**A**) Proliferation was visualized by crystal-violet staining of cells after treatment for 72 hr (top panel). Flow cytometry analysis (bar graph-bottom panel) shows effects on cell cycle after treatment for 24 hr. Percentage of cells in G_0G_1, S, and G_2M phases of the cell cycle is indicated. (**B**) Cells were treated same as in (**A**). EGF was added 15 min prior to harvest. Lysates were subjected to immunoblot analysis with antisera indicated.

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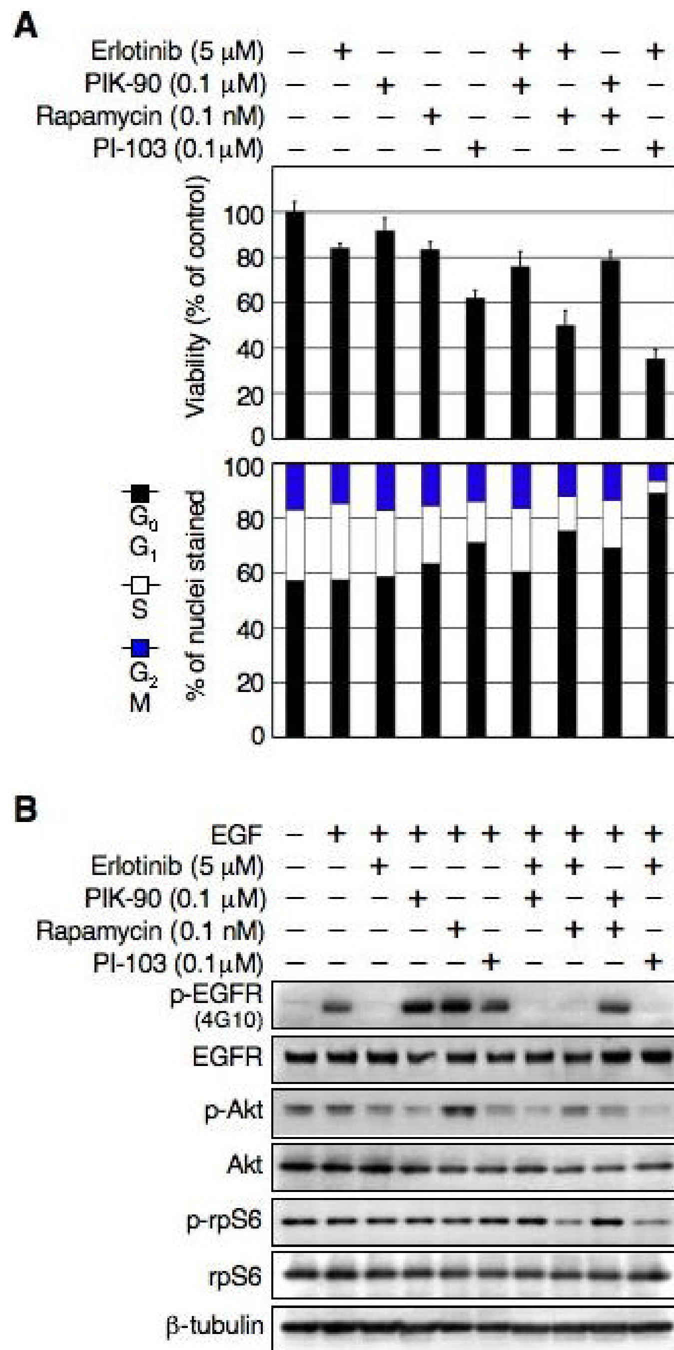

Fig 4.

Cooperative inhibition of EGFR and mTOR are critical to arrest growth of *PTEN*^{mt} human glioma cells. (**A**) U87:*EGFR* (*PTEN*^{mt}) cells were treated with erlotinib, PIK-90, rapamycin, PI-103, erlotinib plus PIK-90, erlotinib plus rapamycin, PIK-90 plus rapamycin, or erlotinib plus PI-103 at dosages indicated. Viable cells were measure by WST-1 assay after treatment for 72 hr. Error was calculated among triplicate measurements (top panel). Flow cytometry analysis (bar graph-bottom panel) shows effects on cell cycle after treatment for 24 hr. Percentage of cells in G_0G_1, S, and G_2M phases of the cell cycle is indicated. (**B**) Cells were treated as in **(A).** EGF was added 15 min prior to harvest. Lysates were subjected to immunoblot analysis with antisera indicated.