Mammalian Target of Rapamycin Inhibition Promotes Response to Epidermal Growth Factor Receptor Kinase Inhibitors in PTEN-Deficient and PTEN-Intact Glioblastoma Cells

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Abstract

The epidermal growth factor receptor (EGFR) is commonly amplified, overexpressed, and mutated in glioblastoma, making it a compelling molecular target for therapy. We have recently shown that coexpression of EGFRvIII and PTEN protein by glioblastoma cells is strongly associated with clinical response to EGFR kinase inhibitor therapy. PTEN loss, by dissociating inhibition of the EGFR from downstream phosphatidylinositol 3-kinase (PI3K) pathway inhibition, seems to act as a resistance factor. Because 40% to 50% of glioblastomas are PTEN deficient, a critical challenge is to identify strategies that promote responsiveness to EGFR kinase inhibitors in patients whose tumors lack PTEN. Here, we show that the mammalian target of rapamycin (mTOR) inhibitor rapamycin enhances the sensitivity of PTEN-deficient tumor cells to the EGFR kinase inhibitor erlotinib. In two isogenic model systems (U87MG glioblastoma cells expressing EGFR, EGFRvIII, and PTEN in relevant combinations, and SF295 glioblastoma cells in which PTEN protein expression has been stably restored), we show that combined EGFR/mTOR kinase inhibition inhibits tumor cell growth and has an additive effect on inhibiting downstream PI3K pathway signaling. We also show that combination therapy provides added benefit in promoting cell death in PTEN-deficient tumor cells. These studies provide strong rationale for combined mTOR/EGFR kinase inhibitor therapy in glioblastoma patients, particularly those with PTEN-deficient tumors. (Cancer Res 2006; 66(16): 7864-9)

Introduction

In cancer, key regulators of intracellular signaling, including receptor tyrosine kinases, are commonly overactive. These persistent signals promote tumor growth and invasion, but may also provide attractive targets for therapy (1). In glioblastoma, the most common malignant primary brain tumor of adults, the epidermal growth factor (EGF) receptor (EGFR) tyrosine kinase is commonly amplified (2, 3). This amplification often occurs in association with expression of a persistently active mutant receptor (EGFRvIII; ref. 3). In early clinical trials, 10% to 20% of malignant glioma patients seem to derive benefit from the EGFR kinase inhibitors erlotinib and gefitinib (4, 5). We have recently shown that coexpression of the EGFRvIII oncogene and the PTEN tumor suppressor protein is strongly associated with clinical response to EGFR kinase inhibitor therapy in two independent sets of malignant glioma patients (6). We also showed that EGFRvIII sensitizes glioblastoma cells to EGFR kinase inhibitors, whereas PTEN loss confers resistance to EGFR tyrosine kinase inhibitors in isogenic cell systems (6). Because glioblastomas frequently lose or have altered PTEN tumor suppressor activity (2, 7, 8), developing strategies to promote clinical response to EGFR kinase inhibitors in these patients is a critical challenge.

PTEN loss promotes resistance to EGFR kinase inhibitors in part by dissociating EGFR inhibition from downstream phosphatidylinositol 3-kinase (PI3K) pathway inhibition (6, 9–11). Therefore, we hypothesized that targeting the PI3K/Akt signaling pathway downstream of PTEN could enhance the sensitivity of PTEN-deficient glioblastoma cells to EGFR kinase inhibitors. Here, we use two isogenic model systems—U87MG glioblastoma cells expressing EGFR, EGFRvIII, and PTEN proteins in relevant combinations, and SF295 glioblastoma cells in which PTEN protein expression has been stably restored—to examine the effect of mammalian target of rapamycin (mTOR) kinase inhibition in promoting response of PTEN-deficient tumor cells to erlotinib.

Materials and Methods

Cell lines and reagents. The human glioblastoma cell line U87MG was purchased from American Type Culture Collection (Rockville, MD), and human SF295 glioblastoma cells were from the NCI-60 cell line panel. All cell lines were routinely maintained in DMEM containing 10% fetal bovine serum. Erlotinib (Tarceva, OSI-774) was kindly provided by Genentech, Inc. (South San Francisco, CA). Rapamycin and human EGF were purchased from Sigma (St. Louis, MO).

Gene construction and retroviral infection. The full-length human PTEN cDNA was cloned into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA), transfected into U87MG and SF295 cells, and single clones resistant to geneticin selection were isolated to generate the stable cell lines U87MG-PTEN and SF295-PTEN.

Human EGFR and EGFRvIII cDNAs were PCR amplified from pLWERNL and pLERNL plasmids and cloned into the retroviral expression vectors

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

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pLPCX and pLHCX, respectively (Clontech, Palo Alto, CA). The resulting constructs pLPCX-EGFR and pLHCX-EGFRvIII were confirmed using automated DNA sequencing. To generate retrovirus, the packaging line 293T was cotransfected with pHIT60, pMDG-VSV-G, and one of either pLPCX, pLPCX-EGFR, or pLHCX-EGFRvIII, using FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN). High-titer viruses were collected 48 hours later and used to infect U87MG or U87-PTEN target cells

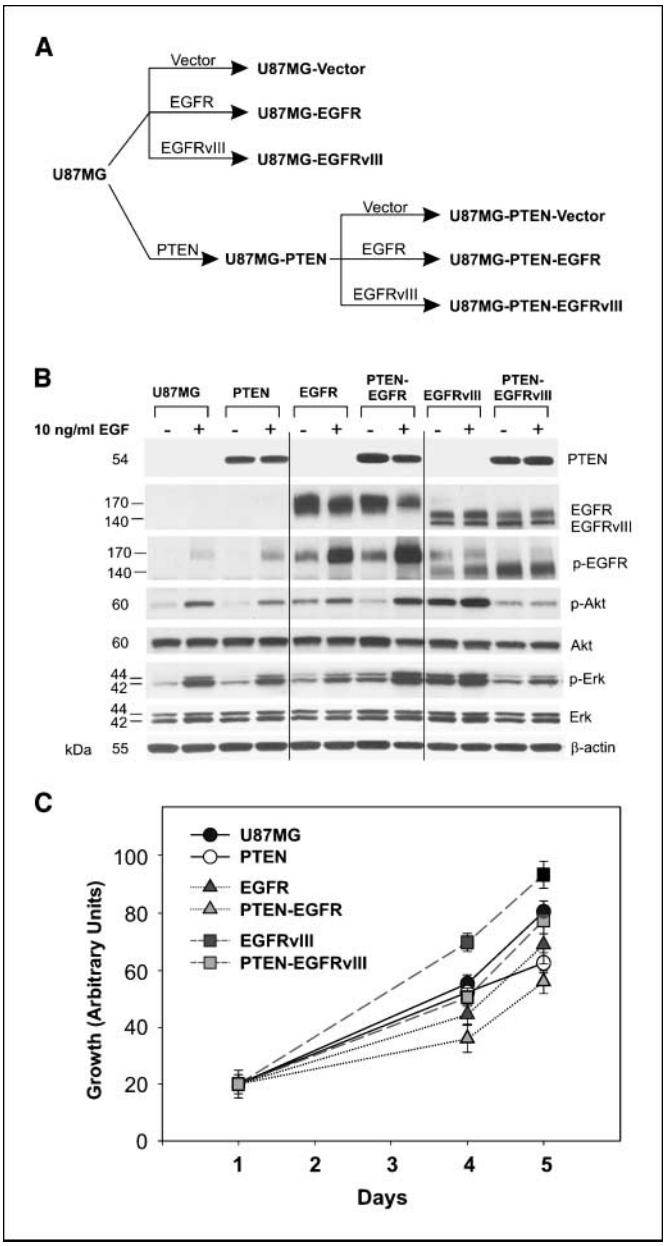

Figure 1. PTEN, EGFR, and EGFRvIII interaction regulates PI3K pathway activation and tumor cell proliferation. *A,* schematic diagram showing the retroviral transduction strategy for generating U87 glioblastoma cells expressing EGFR, EGFRvIII, and PTEN in all relevant combinations. *B,* stimulation of serum-starved cells with 10 ng/mL EGF for 15 minutes induced EGFR phosphorylation and resulted in downstream phosphorylation of Akt and Erk kinases. The ligand-independent mutant EGFRvIII was constitutively phosphorylated and increased baseline Akt and Erk phosphorylation compared with parental U87MG cells, whereas PTEN consistently diminished basal Akt activation, but not Erk, in unstimulated cells. *C,* cell proliferation under serum-free conditions over 5 days was dramatically increased in EGFRvIII-expressing cells compared with parental U87MG cells, whereas PTEN effectively inhibited EGFRvIII-mediated proliferation, as well as proliferation of U87MG cells.

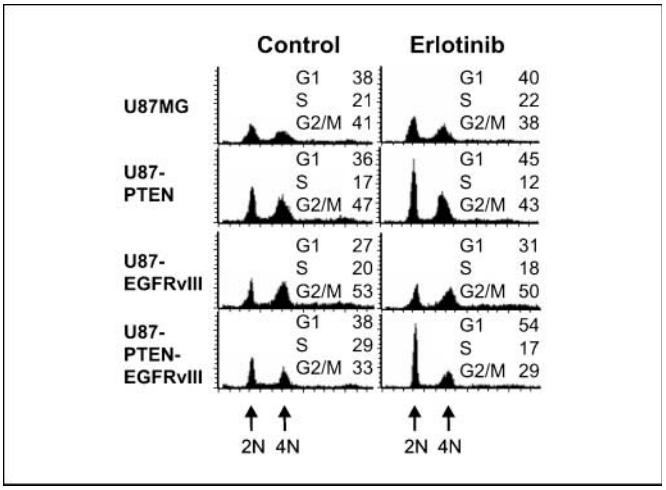

Figure 2. EGFRvIII and PTEN coexpression sensitizes glioblastoma cells to erlotinib. Treatment with 10 μmol/L erlotinib for 48 hours substantially increased the G_1 fraction of U87-PTEN-EGFRvIII cells relative to U87-EGFRvIII cells, and of U87-PTEN cells relative to U87MG cells. Experiments were repeated thrice and representative results from one experiment are presented.

according to standard procedures. Following retroviral infection, single-cell clonal isolates were selected in the presence of puromycin (for pLPCX-EGFR) or hygromycin (for pLHCX-EGFRvIII) for 2 to 4 weeks by limiting dilution, and six clones per cell line were expanded and analyzed for PTEN, EGFR, or EGFRvIII expression by immunoblotting. For each subline, a single clone stably expressing the relevant proteins at steady levels was then selected for use in subsequent experiments.

Immunoblot analysis. Immunoblot analysis was done as previously described (6). Primary antibodies to the following antigens were used: EGFR/EGFRvIII cocktail (Biosource Corp., Camarillo, CA); PTEN (Cascade); phospho-Tyr, Akt, phospho-Akt (Ser^{473}, clone 587F11), S6, phospho-S6 ($Ser^{235/236}$; Cell Signaling Technology, Beverly, MA); and β-tubulin (T4026; Sigma). Horseradish peroxidase–conjugated secondary antibodies were from Cell Signaling Technology.

Cell proliferation assay. Four thousand cells per well were seeded into 96-well plates. Twenty-four hours later, the cells were serum-starved and incubated at 37°C for up to 5 days. At each time point indicated, plates were fixed and stained with 0.25% crystal violet in methanol, and the cells quantified using Alpha Imager 2200 V5.04 (Alpha Innotech Corporation, San Leandro, CA). Alternatively, cell growth under drug treatment was measured using the WST-1 Cell Proliferation Assay kit (Chemicon, Temecula, CA). Briefly, 3,000 cells per well were seeded in a 96-well plate, allowed to adhere overnight, and then incubated with fresh complete medium containing the indicated concentrations of erlotinib, rapamycin, or both for 4 days before measuring growth.

Drug sensitivity assays. One thousand cells were seeded per well of a 96-well plate. Twenty-four hours later, cells were treated with erlotinib in whole medium at various concentrations ranging from 0 to 10 μmol/L in replicates of eight wells per condition. A dose response of erlotinib (0-10 μmol/L) was also conducted in the presence of a constant 0.1 nmol/L rapamycin dose to study the effects of combined treatment. Cells were incubated for 10 to 14 days, stained with crystal violet, and quantified using Alpha Imager 2200 V5.04 software. Background readings for medium alone were subtracted from experimental wells. IC_{50} values were calculated using the software Dose-Effect Analysis with Microcomputers (12). Cell death in the presence or absence of 10 μmol/L erlotinib, 1 nmol/L rapamycin, or a combination of both was determined by trypan blue exclusion. Fifty thousand cells were plated in each well of a six-well plate, allowed to adhere, then treated with inhibitors for 3 to 4 days, at which point the supernatant (containing dead cells) and trypsinized adherent cells were pooled, gently pelleted, and resuspended in trypan blue for cell counting.

Cell cycle analysis. Cells were incubated with or without 10 μmol/L erlotinib for 24 hours, then both adherent and floating cells were harvested and subjected to flow cytometric cell cycle analysis by propidium iodide staining (FACSCalibur Flow Cytometer, Becton Dickinson, Mansfield, MS).

Results and Discussion

PTEN, EGFR, and EGFRvIII: effects on PI3K pathway activation and tumor proliferation. U87MG glioblastoma cells are PTEN deficient, express low levels of wild-type EGFR, and lack EGFRvIII protein (13). We expressed PTEN, EGFR, and EGFRvIII proteins in relevant combinations in parental U87MG cells by retroviral transduction, selecting the following stable cell lines: U87-PTEN, U87-EGFR, U87-PTEN-EGFR, U87-EGFRvIII, and U87-PTEN-EGFRvIII (Fig. 1*A*). Treatment of each cell line with EGF under serum-free conditions stimulated EGFR phosphorylation and promoted downstream Akt and Erk phosphorylation (Fig. 1*B*). EGFRvIII was constitutively phosphorylated and promoted increased baseline levels of Akt and Erk phosphorylation compared with parental cells (Fig. 1*B*). EGF stimulation also enhanced downstream Akt phosphorylation in EGFRvIII-expressing cells, suggesting that stimulation of wild-type EGFR may contribute to PI3K pathway activation in EGFRvIII-expressing cells. PTEN coexpression diminished baseline phosphorylation of Akt, but did not consistently suppress Erk phosphorylation across all of the cell lines (Fig. 1*B*). Thus, the exogenously expressed proteins were biochemically active, and coexpression of PTEN modulated EGFR and EGFRvIII-mediated signaling through the PI3K pathway.

To study the functional consequences of PTEN, EGFR, and EGFRvIII expression in these cells, we examined their effects on cell proliferation. EGFRvIII significantly promoted glioblastoma cell proliferation under serum-free conditions over 5 days (P = 0.01), whereas PTEN coexpression dramatically inhibited EGFRvIII-mediated proliferation (P = 0.00006), as well as proliferation of U87MG cells (P = 0.01; Fig. 1*C*). Wild-type EGFR had much less effect on proliferation under serum-free conditions, which may be consistent with the requirement for ligand stimulation of wild-type EGFR (14). These results indicated that these proteins were functionally active in the tumor cells, and that chronic activation of the PI3K pathway promoted glioblastoma cell proliferation.

Combined EGFR/mTOR inhibition enhances growth arrest and provides additive downstream PI3K pathway inhibition in PTEN-deficient glioblastoma cells. We have previously shown that EGFRvIII and PTEN coexpression in these isogenic U87MG cells rendered them highly susceptible to growth arrest mediated by the

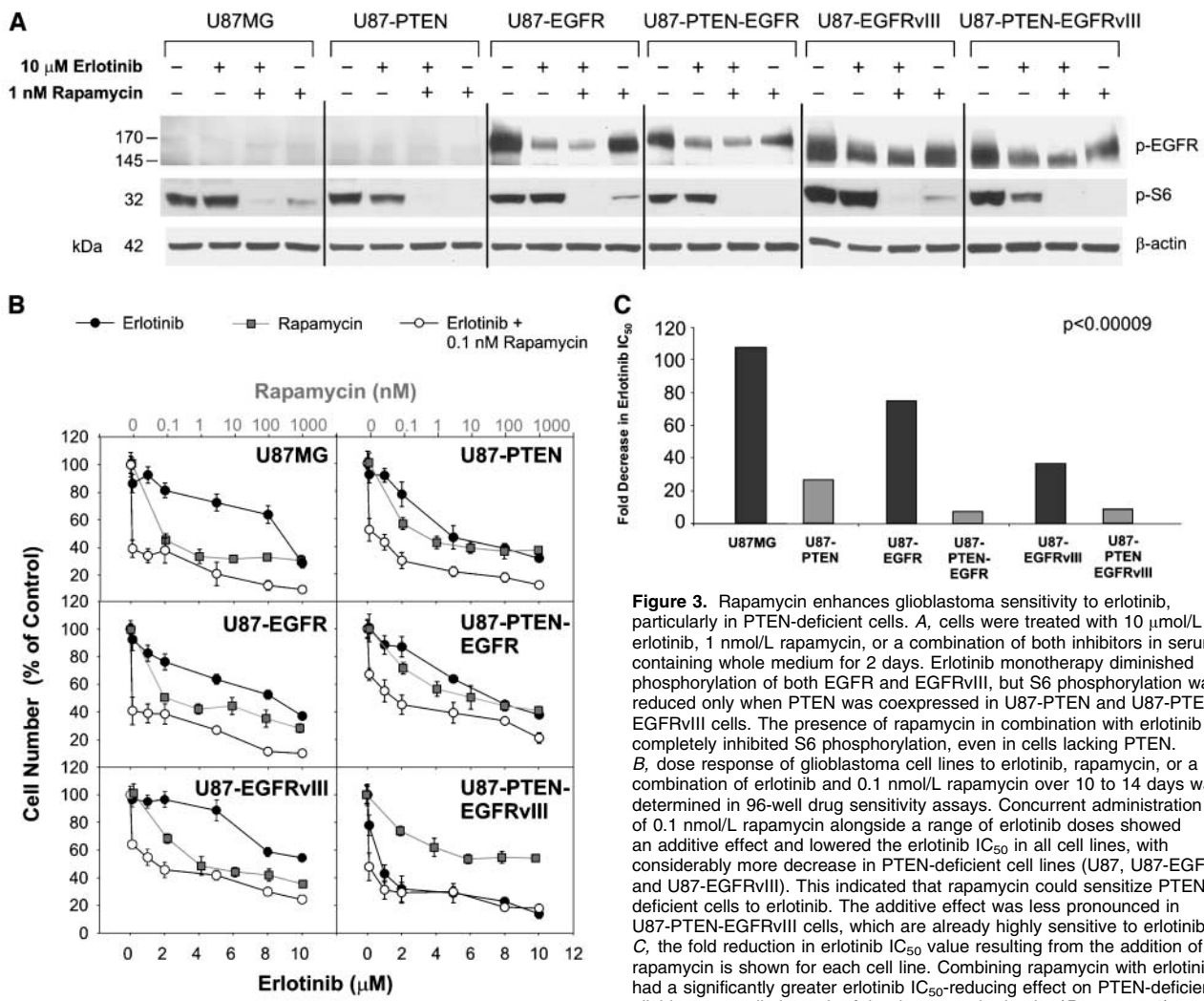

Figure 3. Rapamycin enhances glioblastoma sensitivity to erlotinib, particularly in PTEN-deficient cells. *A,* cells were treated with 10 μmol/L erlotinib, 1 nmol/L rapamycin, or a combination of both inhibitors in serum-containing whole medium for 2 days. Erlotinib monotherapy diminished phosphorylation of both EGFR and EGFRvIII, but S6 phosphorylation was reduced only when PTEN was coexpressed in U87-PTEN and U87-PTEN-EGFRvIII cells. The presence of rapamycin in combination with erlotinib completely inhibited S6 phosphorylation, even in cells lacking PTEN. *B,* dose response of glioblastoma cell lines to erlotinib, rapamycin, or a combination of erlotinib and 0.1 nmol/L rapamycin over 10 to 14 days was determined in 96-well drug sensitivity assays. Concurrent administration of 0.1 nmol/L rapamycin alongside a range of erlotinib doses showed an additive effect and lowered the erlotinib IC_{50} in all cell lines, with considerably more decrease in PTEN-deficient cell lines (U87, U87-EGFR, and U87-EGFRvIII). This indicated that rapamycin could sensitize PTEN-deficient cells to erlotinib. The additive effect was less pronounced in U87-PTEN-EGFRvIII cells, which are already highly sensitive to erlotinib. *C,* the fold reduction in erlotinib IC_{50} value resulting from the addition of rapamycin is shown for each cell line. Combining rapamycin with erlotinib had a significantly greater erlotinib IC_{50}-reducing effect on PTEN-deficient glioblastoma cells in each of the three matched pairs (P = 0.00001).

EGFR kinase inhibitor erlotinib, a result that correlates tightly with what we have observed in patients treated with erlotinib (6). This further supports the functional validity of the U87 isogenic cell line system. The erlotinib IC_{50} values for these cells determined under normal serum-containing growth conditions are shown in Supplementary Table S1. As expected, erlotinib promoted a cytostatic response as measured by a substantial increase in the G_1 fraction of U87-PTEN-EGFRvIII relative to U87-EGFRvIII cells, as well as in U87-PTEN cells relative to U87MG cells (Fig. 2). We detected no evidence of apoptosis by flow cytometry, morphologic analysis, or Western blot analysis of poly(ADP)ribose polymerase cleavage (data not shown). Erlotinib treatment alone inhibited both EGFR and EGFRvIII phosphorylation, but diminished downstream S6 phosphorylation only when PTEN was coexpressed in U87-PTEN and U87-PTEN-EGFRvIII cells (Fig. 3*A*), consistent with cell cycle analysis and our previous findings (6). These results confirm that EGFRvIII/PTEN coexpression, and to a lesser extent PTEN expression alone, sensitizes glioblastoma cells to erlotinib-mediated growth arrest. They also lend support to the hypothesis that PTEN loss promotes resistance to EGFR kinase inhibitors by dissociating EGFR inhibition from downstream PI3K pathway inhibition (6, 9), because inhibition of EGFR or EGFRvIII phosphorylation did not necessarily translate into downstream pathway inhibition.

The mTOR kinase is persistently phosphorylated and thus chronically activated in PTEN-deficient glioblastomas (8), resulting in constitutive activation of the p70S6/4E-BP1 signaling pathway. This mTOR-mediated signal plays a critical role in regulating protein translation and cell proliferation (15). Combined inhibition of mTOR and EGFR signaling by AEE788 (a dual EGFR/VEGFR2 inhibitor) and RAD001 (an mTOR inhibitor) enhanced tumor shrinkage and prolonged survival relative to either treatment alone in a mouse xenograft model of glioblastoma, suggesting the potential benefit of combination therapy (16). Further, a potential synergistic benefit from combined EGFR/mTOR inhibition of glioblastoma cells was recently shown (17). To the best of our knowledge, however, the influence of PTEN on the effects of combined EGFR/mTOR inhibition have yet to be studied using isogenic model systems of PTEN. Because downstream p70S6/4E-BP1 signaling does not seem to be effectively inhibited by EGFR kinase inhibitors in PTEN-deficient glioblastoma cells (Fig. 3*A*; ref. 6), we hypothesized that PTEN-deficient tumor cells would derive enhanced benefit from combined EGFR/mTOR kinase inhibition. To test this, we compared the sensitivity of the U87 isogenic cell lines to erlotinib in the presence of a constant dose of 0.1 nmol/L rapamycin. Concurrent administration of rapamycin significantly lowered the erlotinib IC_{50}, with considerably more decrease in the PTEN-deficient cell lines (average of 74-fold decrease in erlotinib IC_{50}) compared with their PTEN-expressing counterparts (average of 15-fold decrease in erlotinib IC_{50}; Fig. 3*B*; Supplementary Table S1). Within each cell pair, the individual fold reduction in erlotinib IC_{50} due to the addition of rapamycin was significantly greater when PTEN was lacking ($P < 0.00001$; Fig. 3*C*). We also detected additive inhibition of S6 ribosomal protein phosphorylation, the target of p70s6K, concomitant with the decreased cell growth in PTEN-deficient cells treated with both erlotinib and rapamycin (Fig. 3*A*). Thus, PTEN-deficient glioblastoma cells seem to derive the most benefit from combination EGFR/mTOR inhibitor therapy.

Combined EGFR/mTOR inhibition additively promotes growth arrest and cell death in an independent PTEN-deficient glioblastoma cell line model. U87MG cells may not depend on persistent EGFR signaling for survival, because erlotinib

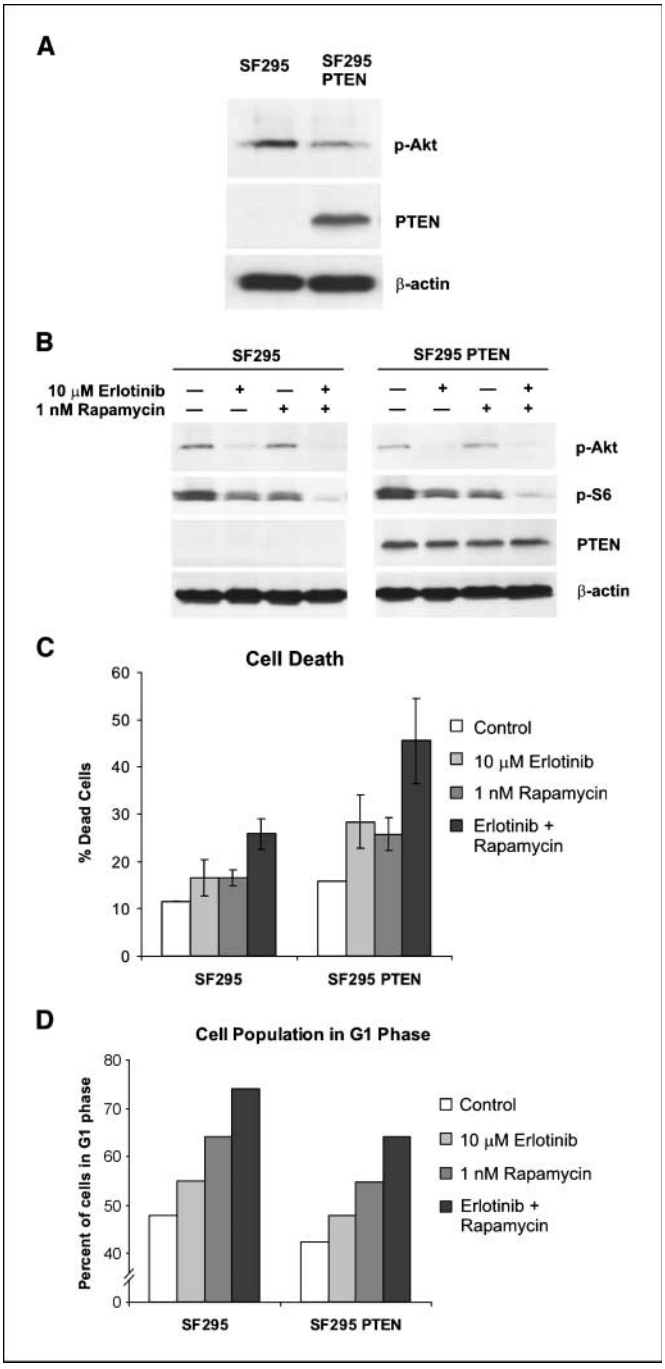

Figure 4. Combined erlotinib and rapamycin treatment induces growth arrest and cell death in PTEN-deficient SF295 glioblastoma cells. *A,* SF295 cells stably expressing PTEN exhibit reduced baseline levels of Akt phosphorylation. *B,* cells were treated with 10 μmol/L erlotinib, 1 nmol/L rapamycin, or a combination of both inhibitors in serum-free medium for 2 days before being stimulated with 10 ng/mL EGF for 15 minutes. Erlotinib (10 μmol/L) strongly inhibited Akt phosphorylation and slightly reduced S6 phosphorylation in both cell lines, whereas 1 nmol/L rapamycin only diminished S6 activation. In contrast, combined erlotinib and rapamycin treatment effectively inhibited both Akt and S6 phosphorylation. *C,* SF295-PTEN cells were significantly more sensitive to erlotinib-mediated cell death than SF295 cells after 3 to 4 days treatment. However, the addition of rapamycin to erlotinib sensitized PTEN-deficient SF295 cells, matching the drug-induced cell death observed in erlotinib-treated SF295-PTEN cells. Combined erlotinib and rapamycin treatment also provided significant additional benefit to SF295-PTEN cells. *D,* the population of cells in G_1 phase was dramatically higher when treated with both erlotinib and rapamycin compared with treatment with either drug alone for 48 hours.

or combined erlotinib/rapamycin treatment induced G_1 arrest, but not apoptosis. Therefore, we analyzed the effect of combined erlotinib/rapamycin treatment on SF295 glioblastoma cells, which undergo apoptosis in the presence of EGFR inhibitors (6). Because SF295 cells lack PTEN, we stably restored PTEN expression by retroviral transduction to study its effects on single or combined therapy. In PTEN-expressing SF295 cells, basal Akt phosphorylation was diminished as expected (Fig. 4*A*), whereas treatment with 10 μmol/L erlotinib alone strongly inhibited Akt phosphorylation, and, to a lesser extent, S6 phosphorylation, in both cell lines (Fig. 4*B*). We have previously shown that PTEN expression markedly enhances the sensitivity of these cells to erlotinib monotherapy (6). Consistent with this previous report and our biochemical data, SF295-PTEN cells showed a significant response to 4-day 10 μmol/L erlotinib monotherapy, with a 25% reduction in proliferation (data not shown) and a 13% increase in cell death compared with control treatment (Fig. 4*C*). In contrast, erlotinib-mediated growth inhibition (14%) and induction of cell death (5%) were clearly diminished in parental SF295 cells lacking PTEN. Rapamycin (1 nmol/L) treatment alone had similar results as erlotinib treatment. These results confirm that PTEN-deficient glioblastoma cells are less sensitive to erlotinib monotherapy compared with those expressing PTEN.

Similar to U87 cells, however, combined treatment with 10 μmol/L erlotinib and 1 nmol/L rapamycin resulted in a substantial additive reduction of S6 phosphorylation (Fig. 4*B*). Likewise, when erlotinib was combined with rapamycin in functional assays, the levels of drug-induced growth inhibition and cell death in PTEN-deficient SF295 cells were considerably enhanced (38% and 14%, respectively), matching or exceeding the effects seen in SF295-PTEN cells treated with erlotinib alone (Fig. 4*C*). Interestingly, PTEN-expressing SF295 cells also responded with significant added benefit to combination therapy, demonstrating a 53% reduction in proliferation (data not shown) and a 30% increase in cell death (Fig. 4*C*) compared with control. It is possible that in these cells, PTEN restoration only partially inhibits the PI3K/Akt pathway. Because baseline levels of phosphorylated S6 were not significantly different despite a lower level of Akt phosphorylation in PTEN-expressing SF295 cells (Fig. 4*A* and *B*), PTEN may be exerting its suppressive effects to other Akt arms more so than on mTOR. If such was the case, the addition of rapamycin even in the presence of PTEN might allow for more complete inhibition of multiple Akt effectors, specifically mTOR. Consistent with these data, combination therapy also promoted an additive effect on G_1 arrest over single therapies in both cell lines (Fig. 4*D*).

In summary, we have shown that rapamycin enhances the sensitivity of PTEN-deficient tumor cells to erlotinib, and that PTEN-intact tumor cells may also derive additional benefit from combination therapy. In two isogenic model systems, we have shown that combined EGFR/mTOR kinase inhibition has an additive effect on inhibiting downstream PI3K pathway signaling and can promote both growth arrest and tumor cell death. Taken together, the consistency of our results across the entire panel of U87 sublines and the completely independent isogenic SF295 glioblastoma cells, coupled with the ability of the U87 sublines to accurately model the effects of erlotinib treatment observed in patients (6), indicate that the results we have observed are not due to clonal effects of the cell line systems. These results highlight the importance of effective inhibition of PI3K pathway signaling in determining the response of glioblastoma cells to EGFR kinase inhibitors. The fact that PTEN-expressing cells were further sensitized to erlotinib by the addition of rapamycin also suggests the possibility that PTEN may influence erlotinib response through both mTOR-dependent and mTOR-independent branches of the PI3K pathway. In some cells, PTEN may elicit erlotinib sensitivity by suppressing mTOR-independent targets and pathways, such that the addition of mTOR inhibition provides additional therapeutic benefit. Moreover, there may be other regulators of mTOR besides Akt because basal S6 phosphorylation was not significantly inhibited in PTEN-expressing SF295 cells despite a measurable decrease in Akt activation. These results are consistent with our previous finding that both Akt-dependent and Akt-independent branches of the PI3K signaling pathway modulate the effects of PTEN loss on sensitivity to EGFR kinase inhibitors (6). As we previously reported, PTEN loss was associated with constitutively higher levels of EGFRvIII phosphorylation (Fig. 3*A*), indicating that PTEN loss may also regulate EGFRvIII phosphorylation itself, independent of Akt and mTOR (6). The results presented here provide a strong mechanistic rationale for combined mTOR/EGFR kinase inhibitor therapy in glioblastoma patients, both for those whose tumors are PTEN-deficient as well as for those in which PTEN is intact.

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