

# Characterization of structurally distinct, isoform-selective phosphoinositide 3'-kinase inhibitors in combination with radiation in the treatment of glioblastoma

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## Abstract

The phosphoinositide 3'-kinase (PI3K)-mediated signaling pathway plays a key role in fundamental cellular functions important in normal cellular homeostasis and malignant transformation. Deregulated signaling through this pathway contributes to development of gliomas and their resistance to radiation and chemotherapy. Targeting the PI3K signaling pathway has thus emerged as a promising approach to successful treatment of gliomas. We assessed the radiosensitizing potential of four small-molecule inhibitors that differ in their activities against specific isoforms of the PI3K 110-kDa catalytic subunit (p110). p110 $\alpha$  inhibitors blocked phosphorylation of both protein kinase B/Akt and S6 in all cell lines examined, effectively decreased cellular proliferation, and produced additive cytotoxic effects in combination with radiation therapy. The p110 $\beta$  inhibitor exhibited limited biochemical effects and failed to decrease cellular proliferation or viability as either a single agent or in combination with radiation or rapamycin. *In vivo* studies examining the effects of the p110 $\alpha$  inhibitor in combination with radiation indicated a signifi-

cant reduction in tumor growth rate induced by the combined treatment compared with each treatment modality alone. This translated into a trend toward prolonged time-to-failure for mice in the combination treatment group. In conclusion, PI3K inhibitors are promising agents in the treatment of glioblastomas, especially when used in combination with ionizing radiation. [Mol Cancer Ther 2008;7(4):841–50]

## Introduction

Glioblastoma is the most aggressive form of malignant brain tumors collectively known as gliomas. Approximately 20,000 people in the United States are diagnosed with gliomas every year, half of them diagnosed with glioblastoma. Effective treatments for gliomas include surgical resection, radiation therapy, and chemotherapy. Despite intense efforts to improve these standard treatment modalities in the past two decades, the median survival of glioblastoma patients has remained less than 1 year for decades (1, 2). Efforts to elucidate molecular mechanisms underlying glioma tumorigenesis and resistance to therapy have shed light on key genetic lesions. Targeted therapies directed against these genetic alterations present a promising approach to glioma treatment.

Several well-characterized genetic alterations identified in gliomas are implicated in malignant transformation, tumor progression, and resistance to therapy. These include inactivation of the tumor suppressor gene p53 (3), inactivation of the P16/pRb/CDK4 pathway (4), and amplification of the *epidermal growth factor receptor* (EGFR) gene (5). Phosphatase and tensin homologue deleted from chromosome 10 (PTEN) exhibits lipid phosphatase activity that negatively regulates phosphoinositide 3'-kinase (PI3K). This tumor suppressor is mutated in ~30% of glioblastomas (6). Loss of PTEN, along with EGFR activation, leads to increased and uncontrolled signaling through PI3K activation (7, 8).

Class 1 PI3K is a heterodimeric membrane lipid kinase consisting of a regulatory subunit (responsible for cellular targeting via protein-protein interactions) and a catalytic 110-kDa subunit (p110), of which there are four isoforms (p110 $\alpha$ , p110 $\beta$ , p110 $\delta$ , and p110 $\gamma$ ; ref. 9). On activation by growth factor-stimulated receptor tyrosine kinases or G protein-coupled receptors, PI3K converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate. Phosphatidylinositol 3,4,5-trisphosphate serves as a second messenger that recruits phosphoinositide-dependent kinase 1 and protein kinase B (PKB)/Akt to the plasma membrane for phosphorylation and activation. PTEN antagonizes the effects of PI3K activation by removing the 3'-phosphate from phosphatidylinositol

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3,4,5-trisphosphate, converting it back to phosphatidylinositol 4,5-bisphosphate. Activated PKB/Akt in turn phosphorylates a variety of substrates to mediate important biological functions, such as cell proliferation and survival (10). Increasing evidence strongly indicates that constitutive activation of PI3K plays an important role in the development of malignant gliomas and their resistance to standard therapies (11, 12). Targeting PI3K therefore presents a promising approach to the treatment of gliomas.

Indeed, several compounds that inhibit PI3K have been identified and used to assess the potential of targeting PI3K in cancer treatment. Foremost among these are wortmannin and LY294002, both of which sensitize human cancer cells to chemotherapy and radiation *in vitro* and *in vivo* (13–15). As radiation constitutes a standard modality in the treatment of malignant gliomas, agents that can increase the effectiveness of radiation are urgently needed to prolong survival for these patients. Our laboratory has recently shown that PKB/Akt is responsible for mediating LY294002-induced radiosensitization in glioma cells (16), a finding that has recently been extended to prostate cancer (17). Despite their efficacy as single agents and their ability to sensitize malignant cells to radiation, toxicity in animals and lack of substrate specificity of both wortmannin and LY294002 preclude their clinical use (15). In addition, due to promiscuity of these inhibitors, interpretation of the mechanisms underlying radiosensitizing functions of these agents remains elusive. The role of PI3K in radiosensitization cannot be clearly defined because related kinases, such as ataxia telangiectasia mutated, mammalian target of rapamycin (mTOR) and DNA-dependent protein kinase, are also inhibited by wortmannin and LY294002.

In this study, we sought to assess the antineoplastic efficacy and radiosensitizing potential of several novel small-molecule PI3K inhibitors that differ in their p110 isoform specificity. These include the pyridinylfuranopyrimidine PI-103, the imidazopyridines PIK-75 and PIK-90, and the morpholinochromone PIK-108. The *in vitro* specificities of these compounds have been characterized previously (18). PI-103 potently inhibits all four class 1 PI3K isoforms as well as mTOR. PIK-90 was included because it inhibits p110 $\alpha$  and p110 $\gamma$  but not mTOR and therefore helps shed light on the contribution of mTOR inhibition to PI-103 efficacy. PIK-108 is a rather selective inhibitor of p110 $\beta$  and p110 $\delta$ , whereas PIK-75 shows selectivity for p110 $\alpha$ , although it also inhibits some protein kinases. All four agents inhibited PI3K activity in a panel of human malignant glioma cells expressing either wild-type or mutant PTEN. PIK-108 failed to inhibit cellular viability either alone or in combination with rapamycin or radiation. In contrast, PI-103 and PIK-90 led to additive cytotoxicity when combined with ionizing radiation *in vitro* and *in vivo*. Importantly, the combined effects of radiation with PI-103 or PIK-90 were more significant in PTEN mutant cells compared with PTEN wild-type cells. In conclusion, the results of this study suggest that the p110 $\alpha$  isoform may play a key role in survival pathways in PTEN mutant glioma cells.

## Materials and Methods

### Isoform-Selective PI3K Inhibitors

PI-103, PIK-75, PIK-90, and PIK-108 were prepared as 20 mmol/L stocks in DMSO (Sigma) and stored as aliquots at  $-20^{\circ}\text{C}$  before use.

### Antibodies

The following antibodies were used in this study: monoclonal rabbit anti-phospho-PKB/Akt (Ser<sup>473</sup>) and anti-phospho-S6 (Ser<sup>235</sup>/Ser<sup>236</sup>; both from Cell Signaling Technology), monoclonal mouse anti- $\beta$ -actin (Sigma), and anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences).

### Cell Lines and Culture Conditions

PTEN wild-type (LN229, hTERT/E6/E7-expressing astrocytes) and mutant (U87 MG, U251 MG, U138 MG, SF126, and SF210) human glioma cell lines were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin and grown at  $37^{\circ}\text{C}$ , 8%  $\text{CO}_2$ . PTEN wild-type SF763 and SF767 human glioma cell lines were maintained in MEM Eagle's with Earle's BSS medium also supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin but grown instead at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ . Primary astrocytes transfected with hTERT were maintained in DMEM supplemented with 15% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin and grown at  $37^{\circ}\text{C}$ , 8%  $\text{CO}_2$ .

### Preparation of Cell Lysate

Fifty-thousand cells were seeded per well in six-well tissue culture plates (Becton Dickinson Labware), allowed to adhere, and treated 24 h later with PIK-108 (0.1, 0.5, 1, 4, and 10  $\mu\text{mol/L}$ ), PI-103 (0.1, 0.5, 1, 2, and 4  $\mu\text{mol/L}$ ), or PIK-75 (0.1, 0.5, 1, 2, and 4  $\mu\text{mol/L}$ ) for 1 h. Cells were then washed with PBS and subsequently harvested by scraping into 40  $\mu\text{L}$  lysis buffer [1% NP40, 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 25 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L  $\text{NaVO}_4$ , and a Complete Mini protease inhibitor tablet per 10 mL (Roche Diagnostics)]. Cells were incubated for 10 min on ice and then centrifuged at 15,000 rpm for 5 min at  $4^{\circ}\text{C}$ . Supernatants were collected and protein concentrations were measured using the Bio-Rad protein assay reagent according to the manufacturer's instructions. Lysates were then mixed with appropriate volumes of 4 $\times$  protein sample buffer [200 mmol/L Tris (pH 6.8), 400 mmol/L DTT, 8% SDS, 0.4% bromophenol blue, and 40% glycerol] and stored at  $-20^{\circ}\text{C}$  before use.

### Western Blotting

For immunodetection of PKB/Akt and S6 phosphorylation, protein lysates (12  $\mu\text{g/sample}$ ) were denatured for 5 min at  $100^{\circ}\text{C}$  and separated by 4% to 20% SDS-PAGE (Invitrogen Life Technologies) followed by electrotransfer to polyvinylidene difluoride membranes. The membranes were blocked at room temperature with 5% (w/v) nonfat dry milk diluted in TBS (pH 7.4) containing 0.1% Tween 20 for 1 h followed by an overnight incubation with rabbit anti-phospho-PKB/Akt (Ser<sup>473</sup>; 1:5,000) or anti-phospho-S6

(Ser<sup>235</sup>/Ser<sup>236</sup>; 1:2,000) antibody at 4°C. The membranes were then further incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:5,000 in 5% milk/TBS-0.1% Tween 20) for 1 h at room temperature. Protein bands were subsequently visualized on Hyperfilm high-performance autoradiography film (Amersham) using the enhanced chemiluminescence Western Blotting Detection reagents (Amersham Biosciences) according to the manufacturer's instructions. To ensure equal protein loading, the membranes were reprobed with mouse anti- $\beta$ -actin (1:4,000). Blots were scanned using Epon Expression 1680 scanner and protein band intensities were quantitated using Odyssey v1.1, 2002 (Li-Cor Biosciences).

#### Cell Viability Assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (Promega). MTS is converted into aqueous soluble formazan by mitochondrial dehydrogenase in viable cells, providing an indication of cell viability. Briefly, 3,000 cells per well were seeded in 96-well microtiter plates and allowed to attach overnight. Increasing concentrations (triplicates) of PI-103, PIK-75, and PIK-108 were then added to the cells. The MTS reagent was added directly to each well (1:5, v/v) 48 h after treatment, and plates were incubated for 1.5 h at 37°C. The absorbance was then read at 490 nm using an automatic plate reader and analyzed using SOFTmax PRO plate reader software v3.1.1 (Molecular Devices). Cell viability was expressed as percent survival relative to untreated controls.

In combination experiments using PI3K inhibitors in conjunction with ionizing radiation, cells were first treated with PI3K inhibitors for 1 h. Plates were then irradiated with 5 Gy at room temperature in a Mark I-68 Cesium 137 irradiator (J.L. Shepherd & Associates).

#### Clonogenic Survival Assay

Clonogenic assays were first used to test inhibitors as single agents. Exponentially growing U251 MG or LN229 cells were treated with escalating doses of PI-103, harvested 24 h later by trypsinization, and plated in six-well plates already containing the appropriate dose of PI-103 as well as lethally irradiated SF188 cells to maximize plating efficiency. Colony formation assays were then done to assess interactions between inhibitor and radiation. Specified numbers of U251 MG or LN229 cells per well were seeded in six-well plates containing a feeder layer of lethally irradiated SF188 cells and treated for 24 h with PI-103 (1.5  $\mu$ mol/L) or DMSO control. The same numbers of cells were plated in the cultures treated with PI-103 and in the cultures not treated with PI-103. Plates were irradiated with specified doses and colony-forming efficiency was determined in the continued presence of inhibitor. Cultures were then incubated for 8 days and colonies of >50 cells were scored. Cell survival measurements were fitted to a linear quadratic mathematical model using the FIT 2.5 program (19, 20). Within each of at least two independent experiments, two to four different dilutions were

made per radiation dose and each dilution was plated in multiples of six.

#### *In vivo* Xenografts and Drug Administration

Xenografts of human glioma cell lines were established by s.c. inoculation of  $5 \times 10^6$  U251 MG cells into the hind legs of BALB/c *nu/nu* athymic mice (Taconic Farms). Mice were monitored according to the protocol approved by the Institutional Animal Care and Use Committee. When tumors reached the size of 50 to 100 mm<sup>3</sup>, mice were randomly assigned to one of four groups consisting of vehicle control (9 animals), radiation alone (10 animals), PI-103 alone (10 animals), and combined PI-103 and radiation (8 animals). We randomized 10 animals per each group; however, for unknown reason, one animal in the control group and two at the combination group either died or lost >15% of their body weight (and therefore had to be sacrificed). The tumor size of those animals was still <50 mm<sup>3</sup>; therefore, these samples were excluded from statistical analyses. PI-103 was formulated as described previously (21) and was administered by i.p. injections (10 mg/kg) five times per week for a total of 8 weeks. One week after treatment initiation, mice were exposed to two fractions of 2 Gy once a week, with each treatment given 24 h after PI-103 administration. For radiation treatments, ketamine/xylazine-anesthetized mice were exposed to  $\gamma$ -irradiation directed at the tumor site, whereas the rest of the body was shielded by a lead jig. Measurements of tumor length (*L*) and width (*W*) were collected and tumor volumes were calculated ( $L \times W^2 / 2$ ) once a week. Animals were euthanized when tumors reached 2,000 mm<sup>3</sup> in size or became severely necrotic.

#### Data Analysis

For the *in vitro* cell viability experiments shown in Fig. 3A and B, the Wilcoxon signed rank test was used to compare treatment with radiation alone and treatment with a combination of radiation and inhibitor (PI-103 for Fig. 3A and PIK-90 for Fig. 3B). We did this statistical analysis for all nine cell lines together as well as for the PTEN wild-type and PTEN mutant cell lines separately.

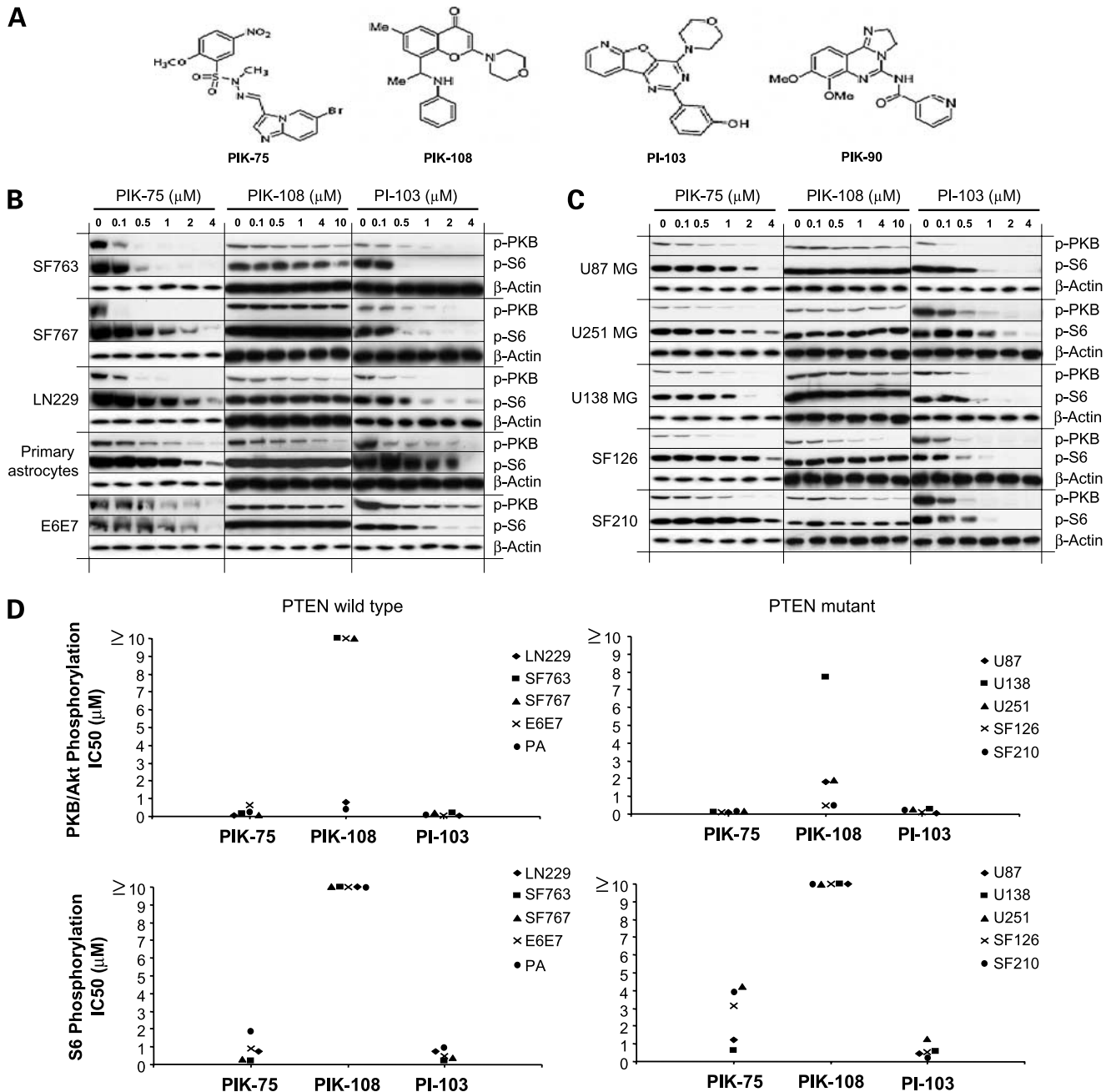
Comparisons of tumor size among treatment groups were done by two-sided *t* test (Microsoft Excel) in which we compared the mean tumor size of the combination treatment with the mean tumor size of each one of the other groups (vehicle control, radiation alone, and PI-103 alone). The analysis was done on day 52 after treatment initiation, because this was the last day on which all animals were still alive. Time to treatment failure (TTF) was defined as a tumor that had reached  $\geq 1,000$  mm<sup>3</sup> in size. TTF was estimated by Kaplan-Meier curves, and differences in TTF between groups were assessed using the log-rank test (GraphPad Prism software, 1992-2005). To be considered a superior treatment, the combination group needed to show significantly better antineoplastic effects than each of the other three groups; therefore, no adjustment was necessary for multiple comparisons. Comparisons of IC<sub>50</sub> values for S6 and PKB phosphorylation between PTEN mutant and PTEN wild-type cell lines was done by two-tailed *t* test (Microsoft Excel).

## Results

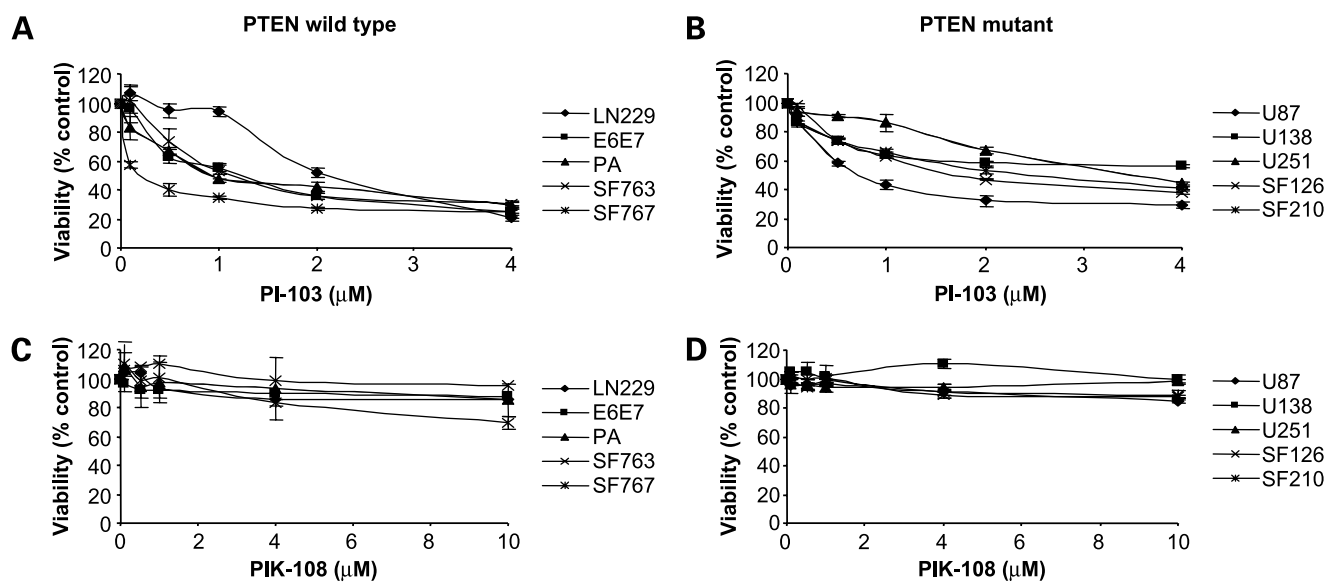
### Isoform-Selective PI3K Inhibitors Exhibit Differential Effects on Downstream Targets of PI3K Activity

To evaluate the effects of PI3K inhibition on downstream signaling, we analyzed the phosphorylation of two well-characterized downstream effectors of PI3K in a panel of

10 glioblastoma cell lines. The panel included five cell lines that express wild-type PTEN, three cell lines that express mutant PTEN, primary human astrocytes that have been immortalized by expression of hTERT, and human primary astrocytes expressing papilloma viral proteins E6 and E7 in addition to hTERT (22). The three PI3K inhibitors chosen



**Figure 1.** Isoform-selective PI3K inhibitors affect PKB/Akt and S6 phosphorylation. **A**, structure of the four PI3K inhibitors used in this study. **B**, Western blots for phospho-PKB/Akt (Ser<sup>473</sup>), phospho-S6 (Ser<sup>235</sup>/Ser<sup>236</sup>), and  $\beta$ -actin in glioma cell lines expressing wild-type PTEN. Primary astrocytes, primary astrocytes containing E6/E7, and gliomas cell lines all expressing wild-type PTEN were assessed by Western blot analyses after 1-h treatment with increasing concentrations of PI3K inhibitors. **C**, Western blots for phospho-PKB/Akt (Ser<sup>473</sup>), phospho-S6 (Ser<sup>235</sup>/Ser<sup>236</sup>), and  $\beta$ -actin in glioma cell lines expressing mutant PTEN. **D**, IC<sub>50</sub> values for each cell line and compound were derived following quantitation of Western blots using Odyssey software.



**Figure 2.** Effects of PI-103 and PIK-108 on the viability of PTEN wild-type and mutant glioma cell lines. Cell viability was assessed using MTS assays. Cells (3,000 per well) were seeded in 96-well microtiter plates and incubated overnight to allow attachment of cells. Increasing doses of PI-103 (**A** and **B**) or PIK-108 (**C** and **D**) were used to treat cohorts of cells expressing wild-type PTEN (**A** and **C**) or mutant PTEN (**B** and **D**). Cells were incubated with drug for 48 h before addition of MTS reagent.

for this screen were PI-103, PIK-75, and PIK-108 (Fig. 1A), whose specificities against lipid and protein kinases have been extensively characterized (18). Phosphospecific antibodies were used to measure expression of Ser<sup>473</sup>-phosphorylated PKB/Akt (a reliable indicator of PKB/Akt activity; ref. 23) and Ser<sup>235</sup>/Ser<sup>236</sup>-phosphorylated ribosomal S6 (sites that are phosphorylated by p70S6K). p70S6K can be activated downstream of PI3K and PKB/Akt through the kinase mTOR, but mTOR and p70S6K can also be regulated independently of PI3K (24). Cells were treated with increasing doses of inhibitors to observe patterns of inhibition of PKB/Akt and S6 phosphorylation in wild-type PTEN and mutant PTEN cell lines (Fig. 1B and C). Extent of inhibition was quantitated and graphed, and the concentrations of each compound required to inhibit PKB/Akt or S6 phosphorylation by 50% were calculated for each cell line. Figure 1D displays these IC<sub>50</sub> values for each compound tested in each cell line.

As expected for a multitargeted PI3K and mTOR inhibitor, PI-103 exhibited equally potent inhibition of PKB and S6 phosphorylation, with IC<sub>50</sub> values of <0.5 μmol/L for both. Similar IC<sub>50</sub> values for this compound were obtained in both wild-type PTEN and mutant PTEN cell lines. PIK-75, a p110α inhibitor that does not inhibit mTOR, also showed comparable potency for PKB/Akt inhibition in PTEN wild-type and mutant cell lines with IC<sub>50</sub> values ranging from 0.1 to 0.6 μmol/L regardless of PTEN status. However, PIK-75 potency for inhibition of S6 phosphorylation appeared greater in the wild-type PTEN-expressing cell lines than in mutant PTEN-expressing cells ( $P = 0.03$ , two-tailed  $t$  test). PIK-108, a p110β inhibitor, showed variable inhibition of PKB/Akt phosphorylation but exhibited a trend toward

reducing PKB/Akt phosphorylation more effectively in mutant PTEN-expressing cell lines than in wild-type PTEN-expressing cell lines ( $P = 0.2$ , two-tailed  $t$  test; Fig. 1B and D). PIK-108 at concentrations up to 10 μmol/L failed to inhibit S6 phosphorylation in all glioma cell lines examined (Fig. 1B-D).

#### Isoform-Selective PI3K Inhibitors Exhibit Differential Effects on Cell Viability

Cell viability following treatment with each agent was analyzed in the context of the isoform specificity of each inhibitor and the PTEN status of each cell line (Fig. 2). PIK-75 showed generalized cytotoxicity and led to ubiquitous cell death in a manner unrelated to its ability to inhibit PI3K (data not shown). Therefore, only PI-103 and PIK-108 were analyzed in cell viability experiments, using MTS assays done following 48 h of drug exposure.

Treatment of all cell lines with PI-103 caused a dose-dependent decrease in cell viability. U87 MG and SF767 were most sensitive to PI-103 treatment, with 50% loss of viability occurring at <1 μmol/L for each cell line (Fig. 2A and B). The remaining cell lines all showed 50% loss of viability at doses ranging from 1 to 4 μmol/L. In contrast, PIK-108 showed little to no biological effects in any glioma cell line examined (Fig. 2C and D). Whereas PI-103 inhibited phosphorylation of both PKB/Akt and S6, PIK-108 inhibited only PKB/Akt phosphorylation. We therefore asked whether combining PIK-108 with an agent that inhibits S6 phosphorylation, such as rapamycin, would now result in cytotoxicity. Although rapamycin caused a moderate growth inhibition, it did not enhance the effects of PIK-108 in any cell line tested (data not shown).

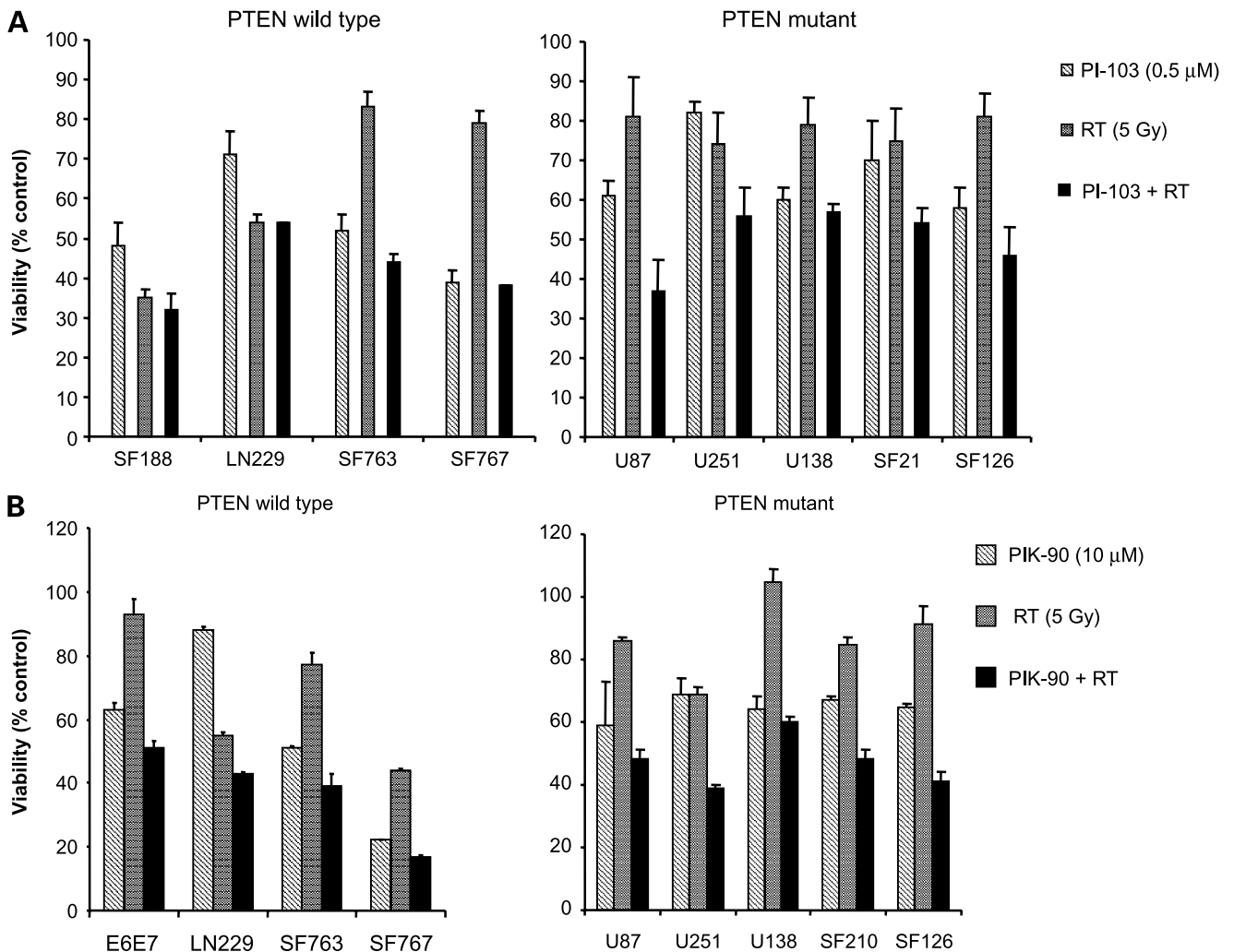
### PI3K Inhibitors Enhance the Cytotoxic Effects of Radiation Preferentially in PTEN Mutant Cells

Recent evidence indicates that deregulated PI3K signaling plays a major role in radiation resistance of many human tumors (25), and we therefore asked whether PI3K inhibitors can sensitize glioblastoma cells to radiation. The potential of using these PI3K inhibitors to overcome radiation resistance led us to test whether each PI3K inhibitor would increase the cytotoxic effects of radiation in this panel of gliomas.

Using MTS assays, we investigated the interactions between radiation and each PI3K inhibitor. PIK-108 failed to potentiate the effects of radiation in any cell line examined (data not shown). In contrast, PI-103 displayed additive cytotoxicity with radiation, an interaction that appeared more pronounced in cell lines expressing mutant

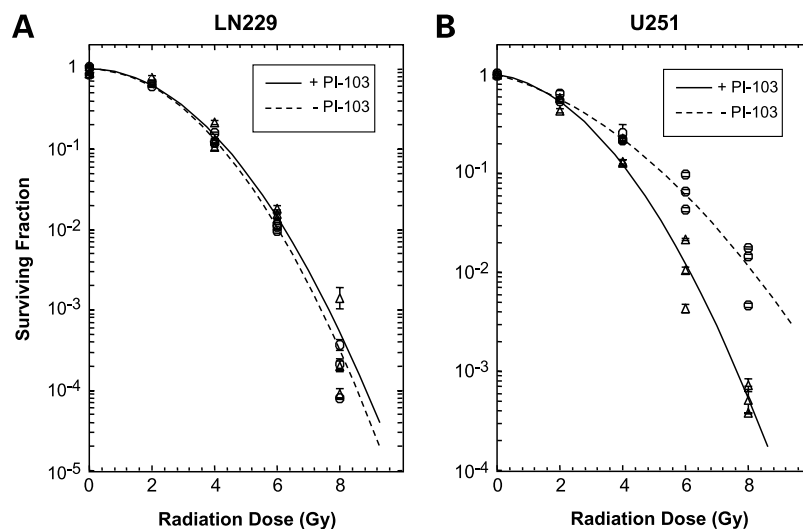
PTEN. None of the wild-type PTEN-expressing cell lines showed additive effects of PI3K inhibition and radiation, whereas all but one mutant PTEN cell line (U183 MG) exhibited increased cytotoxicity when PI-103 was combined with radiation (Fig. 3A). Statistical analysis using the Wilcoxon signed rank test revealed that the combination of radiation and PI-103 was significantly more effective than radiation alone ( $P < 0.01$ , two-sided, with all nine cell lines included). When the five mutant PTEN and four wild-type PTEN cell lines were examined as two separate groups, the benefit afforded by adding PI-103 to radiation was more pronounced in mutant PTEN cells compared with wild-type PTEN cells ( $P = 0.06$  and  $0.25$  for mutant PTEN and wild-type PTEN cell lines, respectively).

Inhibition of mTORC1 has also been shown to enhance the effects of radiation in glioma cells in some systems (26).



**Figure 3.** Combined treatment using PI3K inhibitors and radiation. MTS assays were used to determine cell viability post-treatment with inhibitors and ionizing radiation as a combination treatment modality. Cells (3,000 per well) were seeded in 96-well microtiter plates and treated with the indicated concentration of PI3K inhibitors as carried out in the single-agent MTS experiments. Radiation of cell lines expressing wild-type or mutant PTEN was administered 1 h after treatment with either (A) PI-103 or (B) PIK-90. Cells were then incubated for 48 h before addition of MTS reagent.

**Figure 4.** PI-103 radiosensitizes PTEN mutant-expressing glioblastoma cell line but not wild-type PTEN-expressing cell line. Clonogenic survival assays following combined radiation and PI-103 treatment were done for (A) LN229 cells expressing wild-type PTEN and (B) U251 MG cells expressing mutant PTEN.



As PI-103 inhibits mTOR, in addition to all four class 1 PI3K isoforms, we asked whether the radiation potentiation achieved by PI-103 was due to inhibition of PI3K alone or whether mTOR inhibition contributed to these effects. We therefore used PIK-90, a potent inhibitor of p110 $\alpha$  that fails to inhibit mTOR (18). PIK-90 is effective at inhibiting Akt Ser<sup>473</sup> phosphorylation in both PTEN wild-type and mutant glioma cell lines but as expected is a weak inhibitor of S6 Ser<sup>235</sup>/Ser<sup>236</sup> phosphorylation (Supplementary Fig. S1).<sup>5</sup> Like PI-103, PIK-90 augmented the cytotoxic effects of radiation to a greater degree in mutant PTEN-expressing cells than in wild-type PTEN-expressing cells. Again, U138 MG was an exception in that, despite its mutant PTEN expression, the cytotoxicity of combined treatment with radiation and PIK-90 was not significantly different than the effects of drug treatment alone (Fig. 3B). Statistical analysis using the Wilcoxon signed rank test revealed that combination treatment with radiation and PIK-90 resulted in superior cytotoxicity compared with radiation alone ( $P < 0.01$ , two sided, with all nine cell lines included), a combined effect that again appeared more pronounced in cells expressing mutant PTEN than those expressing wild-type PTEN ( $P = 0.06$  and  $0.12$  for mutant PTEN and wild-type PTEN cell lines, respectively). When different doses of PIK-90 (Supplementary Fig. S2)<sup>5</sup> or PI-103 (data not shown) were combined with radiation, there was no enhanced combination response even at lower doses of drug.

#### Radiation Sensitization of PI-103 in PTEN Mutant Glioblastoma Cells as Measured by Clonogenic Survival

The ability of PI3K inhibitors to enhance the effects of radiation preferentially in PTEN mutant gliomas compared with PTEN wild-type cell lines was further explored using

clonogenic survival assays, the historical gold standard for radiation sensitivity (27). U251 MG cells (PTEN mutant) and LN229 cells (PTEN wild-type) were treated with 1.5  $\mu\text{mol/L}$  PI-103 or DMSO control for 48 h, exposed to specified doses of radiation, and then assayed for sustained clonogenic capacity. PI-103 did not significantly alter the radiation survival curve of the PTEN wild-type LN229 cells. In contrast, PI-103 clearly enhanced the clonogenic radiation sensitivity of U251 MG, the mutant PTEN-expressing cell line (Fig. 4A and B), confirming the ability of PI3K inhibitors to sensitize gliomas to radiation-induced cytotoxicity, particularly in the absence of wild-type PTEN expression.

#### *In vivo* Radiosensitizing Efficacy of PI-103

To extend these promising *in vitro* results, we investigated the *in vivo* radiosensitizing potential of PI-103 in mice xenografts of U251 MG. U251 MG xenografts were grown in the hind legs of *nu/nu* athymic mice and treated with vehicle control, PI-103 alone, radiation alone, or combined PI-103 and radiation treatments. Two radiation fractions were administered 24 h after treatment with PI-103, in accordance with our *in vitro* clonogenic data that showed that such treatment sequences would maximally enhance growth inhibition. Fifty-two days after treatment began, all vehicle control and some PI-103-treated mice required euthanization due to tumors that had grown greater than 2,000  $\text{mm}^3$  or developed severe necrosis. At that time point, mean tumor sizes were  $1,902 \pm 193$ ,  $1,994 \pm 225$ , and  $1,544 \pm 248 \text{ mm}^3$  for control, PI-103, and radiation, respectively, whereas the mean tumor size of the combination PI-103 and radiation group was  $894 \pm 225 \text{ mm}^3$  (Fig. 5A). Statistical analysis (two-sided  $t$  test) revealed significantly smaller tumors in the combination treatment group compared with each of the three other groups ( $P < 0.01$ ,  $P < 0.01$  and  $P = 0.038$  for comparisons to control vehicle, PI-103 alone, and radiation alone, respectively), indicating a significant effect of the combined treatment on tumor growth rate compared with each treatment modality alone.

<sup>5</sup> Supplementary material for this article is available at Molecular Cancer Therapeutics online (<http://mct.aacrjournals.org/>).

TTF was calculated for the first 90 days following treatment commencement using the method of Kaplan-Meier (Fig. 5B), and treatment differences were assessed using the log-rank test. On day 90, the sole remaining animal in any group was in the PI-103 plus radiation group and the tumor had completely regressed, indicating inhibition of tumor growth in 12.5% of combination animals. The data show a trend toward prolonged TTF for mice in the combination treatment group compared with those in the three other groups ( $P = 0.020, 0.003, \text{ and } 0.088$  for comparison with vehicle control, PI-103 alone, and radiation alone, respectively).

## Discussion

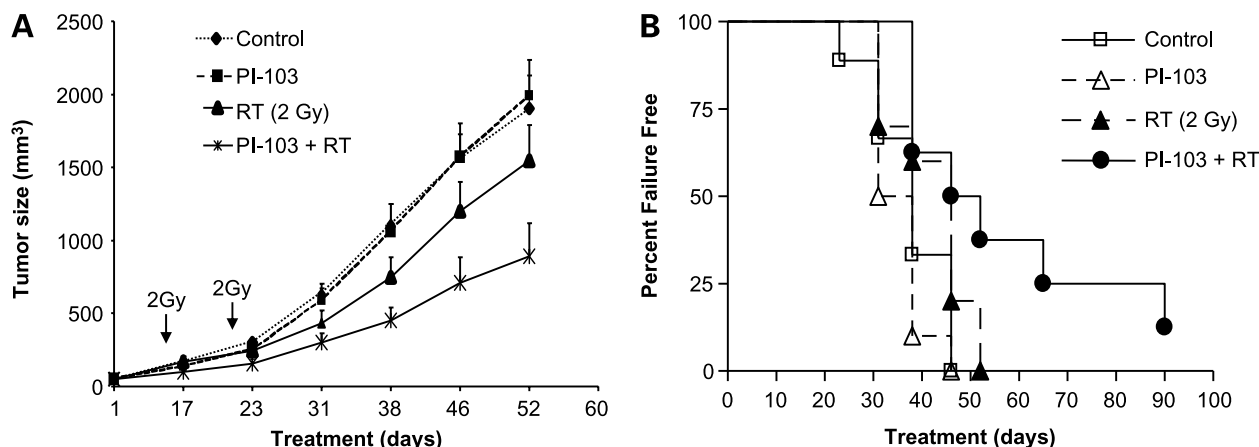
Although radiation is the most effective adjuvant therapy in prolonging life for glioblastoma patients, resistance to radiation invariably precludes cure. Uncontrolled signaling through PI3K plays a critical role in the development of glioblastomas as well as in their resistance to radiotherapy. Until recently, wortmannin and LY294002 were the only available PI3K inhibitors, and although they have facilitated expansion of our knowledge of PI3K signaling, they have limited clinical utility due to their severe toxicities and poor pharmacokinetic properties (15, 28). The marked systemic toxicities of both LY294002 and wortmannin are in large part a result of their lack of specificity and their inhibitory effects on many members of the PI3K superfamily and (18) additional protein kinases (29). These more promiscuous inhibitory functions preclude the use of LY294002 and wortmannin as therapeutic agents and limit their utility in evaluating the roles of specific p110 isoforms.

Recently, novel PI3K inhibitors have been developed and their isoform specificities were profiled. This led to the demonstration that p110 $\alpha$  plays a key role in insulin signaling (using PIK-90; ref. 18), and that combined

inhibition of p110 $\alpha$  and mTOR (using PI-103) inhibits glioblastoma proliferation *in vitro* and *in vivo* (30). In the present study, we characterized the biochemical and biological effects of several structurally distinct, p110 isoform-selective PI3K inhibitors and assessed their efficacy as radiosensitizers. Our results indicate that p110 $\alpha$  is the major isoform involved in PKB/Akt and p70<sup>S6K</sup> phosphorylation in glioblastoma cells and that p110 $\beta$  plays a more restricted role. The role of p110 $\beta$  appears more prominent in PTEN mutant glioma cells, as all PTEN mutant cell lines exhibited inhibition of PKB/Akt phosphorylation by PIK-108, whereas all but one PTEN wild-type glioblastoma cell line (LN229) failed to show phospho-PKB/Akt inhibition by this p110 $\beta$  inhibitor.

The difference in susceptibility of PKB/Akt phosphorylation to p110 $\beta$  inhibition may be exploited therapeutically, with some caveats to be highlighted. First, treatment with PIK-108 (p110 $\beta$  inhibitor) inhibits PKB/Akt phosphorylation not only in wild-type PTEN glioma cell lines but also in normal human astrocytes. Second, although PIK-108 reduces PKB/Akt phosphorylation, it had little to no effect on cell viability as a single agent (Fig. 2C and D) or in combination with rapamycin or radiation (data not shown). Inhibition of p110 $\beta$  not only failed to affect cell viability but also did not decrease S6 phosphorylation, although GSK3 phosphorylation was inhibited concurrently with PKB/Akt (data not shown), as expected.

We propose a model that can account for the observation that the effects of p110 $\beta$  inhibitors on PKB/Akt phosphorylation are more conspicuous in cell lines expressing mutant PTEN. We postulate that PTEN is present at the plasma membrane in close proximity to lipids produced by p110 $\beta$ , and pools of PKB/Akt activated by these lipids only phosphorylate a subset of PKB/Akt substrates with less effect on cell proliferation and/or survival. In cell lines expressing mutant PTEN, in the absence of the lipid



**Figure 5.** PI-103 enhances the antineoplastic effects of radiation and prolongs survival *in vivo*. **A**, U251 MG xenografts grown in athymic mice were treated with (i) vehicle control (daily, i.p. injections, 5 times a week), (ii) 10 mg/kg PI-103 (daily, i.p. injections, 5 times a week), (iii) two doses of 2 Gy  $\gamma$ -radiation administered on the indicated days, or (iv) combined treatment with PI-103 and  $\gamma$ -radiation using the same dosing schedules as used for single agents. Length (L) and width (W) of tumors were measured once a week and volumes were calculated by the formula:  $L \times W^2 / 2$ . **B**, TTF defined as tumors  $>1,000 \text{ mm}^3$  (requiring euthanization of mice) was plotted against time and using the Kaplan-Meier method.

dephosphorylating functions of PTEN, the particular lipid products of p110 $\beta$  play a prominent role, and in that context, the effects of inhibiting p110 $\beta$  are evident. To test this model, we are currently evaluating the phosphorylation states of various PKB/Akt substrates with the hypothesis that inhibitors of p110 $\beta$  and p110 $\alpha$  will decrease phosphorylation of different direct PKB/Akt substrates. For example, TSC2 and PRAS40 are two PKB/Akt substrates that have been implicated in the activation of mTORC1 (31, 32); therefore, our model would predict that they would be preferentially inhibited by p110 $\alpha$  inhibitors compared with p110 $\beta$  inhibitors.

Previous studies from several laboratories, including our own, have used imprecise tools, such as LY294002 and wortmannin, to examine factors responsible for cellular radiation resistance. As a result, various factors, on occasion contradictory, have been reported to influence radiation resistance. LY294002 and wortmannin have been shown to enhance radiation sensitivity and previous studies have been unable to exclude the role of PI3K-like family members in mediating these functions. Ataxia telangiectasia mutated, a PI3K-like family member that is inhibited by LY294002 (33) and wortmannin (34), has been associated with glioma radiation resistance *in vitro* (35). In addition, recent reports show that radioresistance of cancer stem cells within human glioblastoma explants was due to increased activity of Chk1 and Chk2, downstream targets of ataxia telangiectasia mutated. However, because PI-103 and PIK-90 are only weak inhibitors of ataxia telangiectasia mutated, our use of more specific inhibitors of p110 isoforms enables us to rule out ataxia telangiectasia mutated and its downstream effectors as elements that mediate the combined cytotoxicity of p110 $\alpha$  inhibitors and radiation.

In contrast, DNA-dependent protein kinase, another PI3K-like family member, is robustly targeted by PIK-90 and PI-103. Published studies have documented previously the importance of DNA-dependent protein kinase in mediating radioresistance using cells that lack the catalytic subunit Ku-86, a regulatory component of the DNA-dependent protein kinase holoenzyme (36). We therefore cannot exclude a role of DNA-PKB inhibition in the radiosensitizing actions of the inhibitors described in this study.

Perhaps the most compelling data point to a role for PKB/Akt in mediating the radioresistance reported herein. These data consist of studies of the radiation response of a panel of human glioblastoma tumors grown as xenografts in nude mice. In this analysis, 13 xenografts were implanted orthotopically into the right basal ganglia of nude mice, and their survival was monitored following a course of 12 Gy of radiation. Although neither EGFR overexpression nor status of p53 PTEN or p16<sup>INK4a</sup> correlated with response to radiation, tumors with low PKB/Akt phosphorylation were significantly more sensitive to radiation than those with high PKB/Akt phosphorylation (37). This, together with our previous observation that expression of activated PKB/Akt prevented radiosensitization by LY294002 in glioma cells, strongly suggests that PKB/Akt

mediates the combined cytotoxic effects of PI-103 and radiation documented in our current experiments.

In line with our *in vitro* data, we could show that PI-103, although not efficacious as a single agent at a dose of 10 mg/kg, enhances the antineoplastic effects of radiation in U251 MG xenografts *in vivo*. The mean size of tumors exposed to both PI-103 and radiation was significantly smaller than the mean size of tumors treated with vehicle control, PI-103 alone, or radiation alone. This inhibition in tumor growth translated into a trend toward increased TTF in mice treated with the combination regimen, shedding more light on the potential radiosensitizing effect gained through lowering PKB activity.

PI3K inhibitors are now entering clinical practice in the treatment of multiple human malignancies, foremost among them are gliomas. The experiments presented in this and other studies should help guide the most appropriate use of these agents either alone or in combination with radiation.

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