Class I PI3 Kinase Inhibition by the Pyridinylfuranopyrimidine Inhibitor PI-103 Enhances Tumor Radiosensitivity

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Abstract

Cell signaling initiated at the epidermal growth factor receptor (EGFR), RAS oncoproteins, or PI3K contributes to a common pathway that promotes tumor survival after radiation-induced DNA damage. Inhibition of signaling at the level of EGFR, RAS, and PI3K has been tested, but clinical applicability has been shown only at the level of the EGFR or by inhibiting RAS indirectly with prenyltransferase inhibitors. Inhibition of PI3K with LY294002 or wortmannin lacks specificity and has shown unacceptable toxicity in preclinical studies. We previously showed that inhibiting class I PI3K expression with siRNA resulted in enhanced radiation killing of tumor cells. Here, we tested the possibility of achieving specific tumor cell radiosensitization with a pharmacologic inhibitor of class I PI3K, the pyridinylfuranopyrimidine inhibitor PI-103. Our results show that inhibiting PI3K activity reduces phosphorylation of AKT at serine 473. Reduced survival is seen in cells with AKT activation and seems preferential for tumor cells over cells in which AKT activity is not elevated. Reduced survival is accompanied by persistence of DNA damage as evidenced by persistence of γH2AX and Rad 51 foci after irradiation in the presence of the inhibitor. Reduced survival does not result from cell cycle redistribution during the PI-103 treatment intervals tested, although combining PI-103 treatment with radiation enhances the G_2-M delay observed after irradiation. These results indicate that pharmacologic inhibitors with enhanced specificity for class I PI3K may be of benefit when combined with radiotherapy. [Cancer Res 2008;68(14):5915–23]

Introduction

Cellular transformation is frequently accompanied by activation of pathways that promote survival after DNA damage. This activation can occur as a result of oncogenic mutations in genes, such as *ras*, or activation of tyrosine kinase growth factor receptors such as the epidermal growth factor receptor (EGFR). Loss or silencing of negative regulators of survival signals such as the PTEN lipid phosphatase can also promote survival of tumors after DNA damage by promoting AKT activation. Constitutive signaling activated by these lesions is specific to tumors and is not present in normal tissues. AKT activation in head and neck squamous cell cancers, non–small cell lung cancers, and cervical cancers has been associated with poor prognosis (1–3). Our studies are based on the prediction that inhibition of survival signaling will preferentially affect the survival of tumor cells after exposure to DNA damage.

EGFR activation is known to contribute to tumor survival and therapy resistance (4). In the clinic, EGFR inhibition with cetuximab was shown to be beneficial to treatment outcome when combined with radiation (5). Our past studies and the work from other groups defined a pathway that contributes to the radiation survival of many tumor types (6, 7). This pathway includes EGFR, RAS, phosphatidylinositol-3-kinases (PI3K), and AKT. The pathway can be activated by mutation or overexpression at each of these points, as well as at the negative regulator of PI3K signaling, the PTEN phosphatase. We have shown that activation of RAS or PI3K can promote tumor radiation survival (8, 9), and that inhibiting signaling at the level of each of these proteins can decrease tumor cell survival after ionizing radiation exposure (9). Inhibition of this pathway seems to have a specific effect on tumor cells and has been well-tolerated in clinical trials combining inhibitors with radiation (5, 10). The pathway thus poses an attractive target for enhancing the therapeutic window for tumor killing by radiation.

The number of possible activation points in the survival signaling pathway leads to the prediction that inhibition at the level of PI3K may be effective in a wider range of tumors than inhibition at the level of the EGFR. For instance, inhibition at the level of PI3K should be effective against tumors with activation either of EGFR, RAS, or PI3K, whereas inhibition of EGFR signaling might not affect the sensitivity of a tumor with activation of survival signaling at a point downstream of the EGFR. For this reason, we are investigating pharmacologic inhibition of PI3K as a strategy for tumor radiosensitization.

Class IA PI3K are a family of heterodimeric lipid kinases that regulate many cellular processes (11). Autophosphorylation of activated receptor tyrosine kinases activates PI3K by promoting binding of the PI3K regulatory subunits p85α and p85β (12, 13). PI3Ks are also activated by binding to RAS-GTP (14). The p110 catalytic subunits phosphorylate the PI3K substrate (PtdIns(4,5)P2) on the N3 position of the inositol ring, generating PtdIns(3,4,5)P3 (11). PtdIns(3,4,5)P3 binds to proteins with pleckstrin homology domains including protein kinase B/AKT, resulting in activation of this protein, which is known to promote cell survival. There is strong evidence that the PI3K pathway is deregulated in many human cancers (15, 16), and missense mutations have been identified in *PIK3CA*, the gene that encodes the catalytic subunit p110α. Studies using gene targeting of isogenic pairs of colorectal cancers cell lines with either mutant or wild-type (wt) *PIK3CA* showed that the mutation causes constitutive activation of PI3K facilitating metastasis, inhibiting

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apoptosis and producing growth advantage in conditions where growth factors are limiting (17). Our studies have shown that knocking down the expression of specific PI3K or AKT isoforms is effective in reducing tumor clonogenic survival after irradiation in tumors with EGFR overexpression, or *ras* mutation. Genetic inhibition combining siRNAs specific for PI3K p110 and p85 reduced AKT phosphorylation and radiation survival in these cells. Similarly, specific knockdown of AKT-1 expression reduced tumor cell radiation survival (18). These results prompted us to investigate pharmacologic inhibitors of class I PI3K as potential radiation sensitizers.

The effects of inhibiting PI3K have been difficult to evaluate because both of the previously available inhibitors, the fungal metabolite wortmannin and LY294002, showed a broad range of specificity (19–22). In addition, use of these inhibitors was limited to preclinical studies by concerns over toxicity (23, 24). Recently developed inhibitors have been designed to have a narrower range of specificity, thus offering better tools for evaluating the effects of inhibiting PI3K. Such inhibitors would also have enhanced clinical potential. However, careful examination of the specificity of many of these new inhibitors has shown that their specificity in *in vitro* enzyme inhibition assays is also broader than originally anticipated (25). This raises the possibility that off-target inhibition of other kinases could constrain their use as radiosensitizing agents if these off-target effects are important to normal cell radiation responses such as DNA repair. We have examined the effect of the inhibitor PI-103 (26), one of the most specific kinase inhibitors available (27), on the radiosensitivity of a panel of tumors with survival pathway activation at the level of EGFR, RAS, and PI3K. We have also tested whether there is a sufficiently differential effect on the radiosensitivity of tumor cells relative to immortalized and short-term passage primary cell cultures to justify further radiation studies with inhibitors that share the inhibitory spectrum of PI-103.

Materials and Methods

Cells and cell culture. HCT116 and DLD-1 parental tumor cell lines and derivatives of these cell lines homozygous for either wt or activated p110α (17) were kindly provided by Bert Vogelstein (John Hopkins, Baltimore, MD). All other cell lines were obtained from the American Type Culture Collection. Cells were cultured in DMEM containing 4.5 g/L glucose (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), penicillin (100 units/mL), and streptomycin (100 mg/mL; Invitrogen). POCp6 cells were tested in both 10% and 20% serum. All cultures were maintained at 37°C in water saturated 5% CO_2/95% air. Cells were regularly tested to ensure absence of Mycoplasma contamination (MycoAlert; Cambrex).

Inhibitors and inhibitor treatment. PI-103 was obtained from Piramid Pharma under material transfer agreement. LY294002 was purchased from Sigma. All inhibitors were dissolved as concentrated stock solutions in DMSO and stored at −80°C. At the time of use, inhibitors were diluted with culture medium to the indicated concentrations. Control cells were treated with medium containing DMSO at a concentration equivalent to the highest dose used in inhibitor-treated cells.

Inhibitors were added to mid-log phase cell cultures in culture medium at the indicated time and concentrations. For proliferation and cytotoxicity experiments, inhibitor treatment was continued throughout the experiment or washed out after 24 h of exposure as indicated. For clonogenic survival studies, inhibitor treatment was initiated 1 h before irradiation and maintained for 24 h. After the treatment interval, the medium was replaced with drug-free medium. Control cultures underwent medium replacement at the same time to control for this manipulation.

Clonogenic survival curves. In all clonogenic survival experiments, cells were plated from single-cell suspensions and allowed to adhere to culture dishes before irradiation and/or inhibitor exposure. Cells were irradiated with a Mark 1 cesium irradiator (J.L. Shepherd) at a dose rate of 1.7 Gy/min. Alternatively, cells were irradiated with X-rays using an RS-225 X-ray cabinet (Gulmay Medical Ltd) at a dose rate of 2.77 Gy/min. Assays replicated on both irradiators showed no significant differences in the results obtained. Colonies were stained and counted 10 to 30 d after irradiation. Colony counting was primarily accomplished using an Oxford Optronics Colcount. Some primary cells formed diffuse colonies and required manual scoring. The surviving fraction was derived using the formula:

$$(\#Colonies/\#\ of\ cells\ plated)_{irradiated}/(\#Colonies/\#\ of\ cells\ plated)_{unirradiated}.$$

Each point on the survival curve represents the mean surviving fraction from at least three dishes. Clonogenic survival curves are representative of independent replicate experiments.

Analysis of protein phosphorylation. Cells were lysed on culture dishes at the times indicated after PI-103 addition with reducing Triton lysis buffer after rinsing once with PBS. Samples were boiled, sheared, clarified by centrifugation at 14,000 rpm, and stored at −80°C. Protein concentration in lysates was determined by Bicinchoninic acid or Bradford assay. Equal amounts of protein were separated on Invitrogen precast gels and blotted onto nitrocellulose membranes (GE-Amersham). Membranes were blocked in TBS containing 0.1% Tween 20 and 5% nonfat powdered milk. For probing with phospho-specific antibodies, blocking was done using 5% bovine serum albumin (BSA) before the addition of primary antibody. Phospho-AKT (ser-473) and Pan AKT antibody (Upstate) were used at a 1:1,000 dilution. β-actin clone AC-15 (Sigma) was used at a 1:4,000 dilution. Antibody to phospho-p70S6 kinase (Thr412; Upstate Biologicals) was used at a concentration of 0.5 μg/mL. Antibodies to phospho–mitogen-activated protein kinase (MAPK; extracellular signal-regulated kinase 1/2 Thr202/Tyr204; Cell Signaling Technologies) and phospho-DNA-PKcs (S2056; Abcam) were used at a final concentration of 1:1,000. Antibody binding was detected using the enhanced chemiluminescence kit (GE-Amersham). Exposed film was digitized and figures were assembled using Adobe Photoshop and Microsoft PowerPoint.

Analysis of γH2AX and Rad 51 foci. Cells were plated into 96-well plates at a density of 10,000 cells per well and incubated overnight at 37°C with 5% CO_2 to allow cells to attain growth in mid-log phase before drug treatment. Cells were exposed to inhibitor 1 h before irradiation using an RS-225 X-ray cabinet at a dose rate of 2.77 Gy/min (Gulmay Medical Ltd) or IBL 637 cesium irradiator at a dose rate of 1.1 Gy/min (CIS Bio International). The results with these irradiation protocols were equivalent. Cells were fixed with 3% paraformaldehyde diluted in PBS at the specified time points postirradiation and were subsequently permeabilized and blocked with 0.1% Triton (vol:vol) diluted in PBS containing 1% BSA (Sigma) for 1 h at 4°C. Cells were incubated with a primary mouse monoclonal antibody to γ-H2AX (Millipore) or rabbit anti human Rad-51 antiserum (Santa Cruz Biotech.) 1:1,500 dilution overnight at 4°C and subsequently washed thrice in PBS before incubation with secondary antibody for 1 h at room temperature. For γ-H2AX, the secondary antibody was a goat anti-mouse Alexafluor 488 conjugate secondary antibody (Invitrogen) 1:1,200. For detecting Rad-51 staining, a goat anti-rabbit Alexa-568 antibody (1:1,200) was used. Cells were again washed thrice with PBS for 5 min before 4′,6-diamidino-2-phenylindole (DAPI) staining, 0.5 μg/mL diluted with PBS, for 10 min. The DAPI was replaced with PBS before foci were detected using an IN Cell Analyzer 1000 automated epifluorescence microscope (GE Healthcare) with the following excitation and emission filters (Chroma): DAPI—excitation filter, 360 nm (D360_40x); emission filter, 460 nm (HQ460_40M); dichroic mirror, 61002bs. Alexafluor 488—excitation filter, 480 nm (HQ480_40x); emission filter, 535 nm (HQ535_50M);Dichroic mirror, 61002bs. The quantitation of mitotic cell frequency after inhibitor + radiation treatment was done using images of DAPI-stained cells acquired with this automated epifluorescence microscope. Foci and mitotic frequency quantitation was accomplished using IN Cell Analyzer Workstation software (v3.5).

Flow cytometric analysis. Cells were plated onto 60-mm tissue culture dishes (Falcon) and incubated overnight to allow cells to reach mid-log

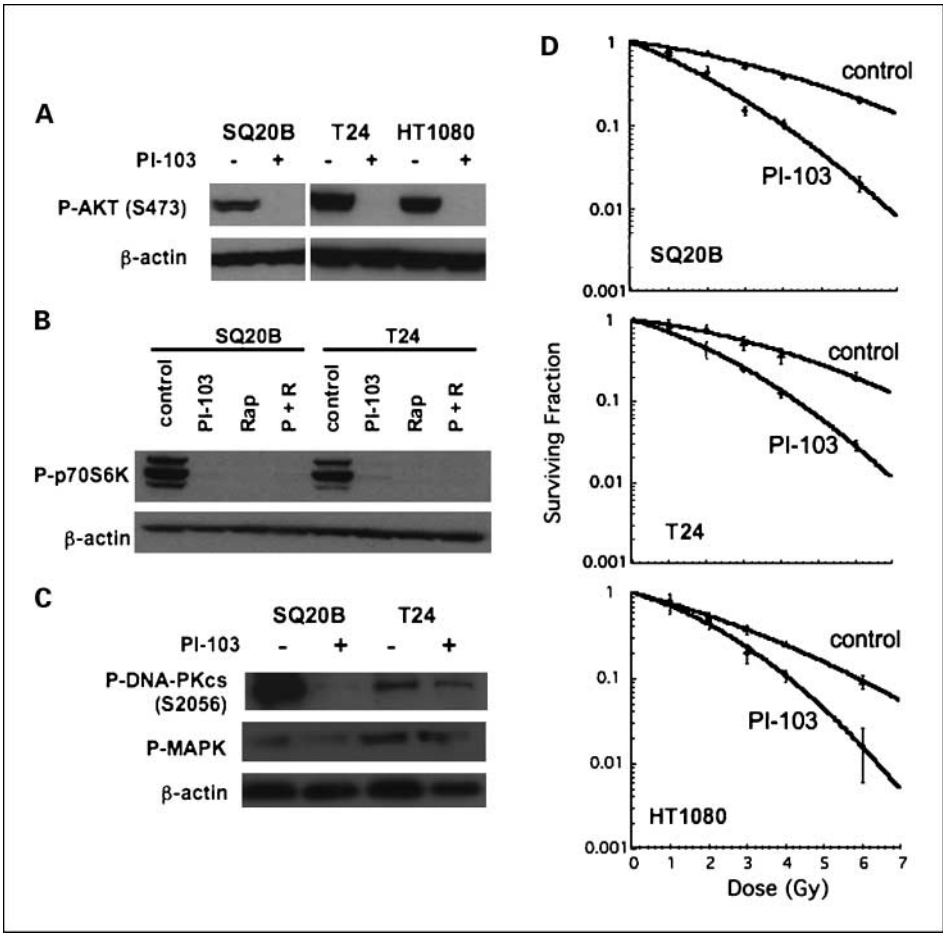

Figure 1. PI-103 inhibits AKT and p70S6 kinase phosphorylation and reduces the clonogenic survival of tumor cells with EGFR or RAS activation. *A,* SQ20B HT1080 and T24 cells were treated with 0.4 μmol/L PI-103 or DMSO (control lanes) and were harvested after 90 min of drug exposure. *B,* SQ20B and T24 cells were treated with 0.4 μmol/L PI-103, 0.1 μmol/L rapamycin (*RAP*), both inhibitors (*P+R*), or DMSO (control lanes), as indicated, and were harvested after 90 min of drug exposure. *C,* SQ20B and T24 cells were treated with 0.4 μmol/L PI-103 or DMSO (control lanes), as indicated, and were harvested after 90 min of drug exposure. For *A, B,* and *C,* cells were lysed in the culture dishes and the lysates separated by electrophoresis and blotted with antibody to AKT (phospho-Ser-473), p70S6 kinase (phospho-Thr412), or DNA-PKcs (phospho-Ser-2056) and phospho-MAPK as described in Materials and Methods. Blots were also probed with antibody to β-actin to validate equal loading. *D,* cells in log-phase culture were harvested and defined cell numbers plated as single-cell suspensions. Cells were allowed to adhere then treated for 1 h with PI-103 (0.4 μmol/L) before irradiation. Twenty-four hours later, the culture medium was replaced with drug-free medium and cells cultured for a further 2 wk before staining for colony formation. Results are representative of at least two experiments; *points,* mean derived from a minimum of three dishes within an experiment; *bars,* SD within replicates.

phase growth before inhibitor treatment. Cells were treated with inhibitor 1 h before irradiation with X-rays using an RS-225 X-ray cabinet (Gulmay Medical Ltd) at a dose rate of 2.77 Gy/min. Cells were trypsinized at the specified time points postirradiation using 0.5% Trypsin/EDTA (Life Technologies-Bethesda Research Laboratories). Cell suspensions were centrifuged at 1,100 rpm, washed with PBS, and resuspended in 1 mL ice cold 70% ethanol and stored overnight at −20°C. Cells were subsequently centrifuged at 1,100 rpm for 10 min and washed with PBS before incubation with 200 μg/mL RNase A diluted in PBS containing 50 μg/mL propidium iodide, for 30 min at room temperature. Cells were protected from light before analysis, which was performed within 2 h of staining. Cytometry and analysis was accomplished using a Becton Dickinson FACSort with Modfit LT analysis software. Data are representative of three independent experiments and were repeated after two and four Gy irradiation.

PI-103 activity assays. *In vitro* PI3K activity was analyzed as described (28), with minor modifications. Briefly, the immune complexes were prepared by incubating 400 μg of proteins with 5 μL of anti-p110α antibody (Santa Cruz Biotechnology) for 2 h at 4°C, followed by 12 h of incubation with Protein A-agarose. The beads were washed thrice with the lysis buffer and once with TNE [10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, and 1 mmol/L EDTA]. Subsequently, the immune complexes were incubated with 50 μL of kinase reaction buffer containing 20 mmol/L HEPES (pH 7.5), 10 mmol/L $MgCl_2$, 200 μg/μ mL phosphatidylinositol (sonicated), 60 μmol/L ATP, and 200 μCi/μ mL [γ-32P]ATP for 5 min at room temperature. The reaction was terminated by adding 80 μL of 1 N HCl, and the phosphorylated lipids were extracted with 160 μL of chloroform methanol mixture (1:1). After samples had been dried down, they were dissolved in chloroform and spotted onto Silica Gel 60 TLC plates (Merck KGaA). Protein kinase assays were performed using a filter-based radiometric platform in the presence of 3 μmol/L PI-103 and 10 μmol/L ATP. Assays were undertaken at Millipore Corp.

Results

PI-103 inhibits PI3K activity and reduces AKT phosphorylation. The inhibitory spectrum of PI-103 has been assessed and previously reported by others (22, 25–27, 29). As a verification of PI-103 activity, the inhibition of PI3K by PI-103 was assessed by kinase assay. Immunoprecipitation of PI3K from SQ20B cells followed by monitoring *in vitro* phosphorylation of phosphatidylinositol in the presence of the inhibitor showed that PI-103 blocked PIP3 production in a dose-dependent manner (data not shown). Because inhibition of PI3K reduces AKT phosphorylation, AKT phospho-Ser-473 was monitored after PI-103 treatment of intact cells by Western blotting of treated cell lysates in all subsequent experiments. Preliminary dose response studies showed that significant inhibition of AKT phosphorylation occurred within 1 h at a dose of 0.4 μmol/L in SQ20B, T24, and HT1080 human tumor cell lines (Fig. 1*A*; data not shown). Because PI-103 was reported to block mammalian target of rapamycin (mTOR) activity (29), we tested whether we could detect this inhibition in T24 and SQ20B cells. Phosphorylation of the mTOR substrate p70S6 kinase was completely inhibited after 1 hour of treatment with 0.4μmol/L PI-103 (Fig. 1*B*). The effect of inhibition by PI-103 on p70S6 kinase phosphorylation was equivalent to that seen with 0.1 μmol/L rapamycin. PI-103 also inhibited DNA-PK phosphorylation in SQ20B, but this effect was

not complete in T24 cells (Fig. 1*C*). MAPK phosphorylation was not significantly affected in either cell. The inhibitory spectrum of PI-103 for other kinases was tested by enzyme inhibition assay. With the exception of c-Raf (54% of control), Lck (14% of control), and Rsk-1 (41% of control), inhibition of kinase enzymatic activity is absent or minimal (<30%) in the presence of 3 μmol/L PI-103 (Supplementary Table S1). The binding (Kd) of PI-103 to PI3K α catalytic domain (1.5 nmol/L) was recently reported to be significantly greater than to Raf (3,700 nmol/L) and Lck (>10,000 nmol/L) catalytic domains (27).

Continuous exposure to PI-103 inhibits tumor cell clonogenicity. The effect of PI-103 on tumor cell proliferation was measured both during continuous treatment and after transient exposure for 24 hours. The reduction in tumor cell survival after continuous exposure to varying concentrations of PI-103 resulted in a dose-dependent inhibition of tumor clonogens (Supplementary Fig. S1). Tumor cells showed differing susceptibilities to this inhibitor. PC3 prostate tumor cells, which are mutant for PTEN, were the most sensitive with a reduction to <20% of control growth at 0.1 μmol/L. Reduction of SQ20B growth was not as great at this dose but was almost complete at 0.4 μmol/L. HCT116 colon cancer cell growth was also inhibited but retained clonogenic potential at the highest dose tested (800 nmol/L).

Although continuous exposure inhibited tumor clonogens, transient exposure of tumor and immortalized cells to 0.4 μmol/L PI-103 for 24 hours did not result in significant cytotoxicity or growth arrest in most cell lines as determined by clonogenic survival (Supplementary Table S2). Because transient exposure to 0.4 μmol/L PI-103 resulted in inhibition of AKT phosphorylation, while permitting the clonal outgrowth of cells without a significant reduction in plating efficiency, this exposure was used for subsequent determinations of clonogenic survival after irradiation.

Inhibition of class I PI3K activity with PI-103 reduces radiation survival of tumor cells with EGFR overexpression or RAS mutation. To determine the effect of PI-103 treatment on radiation sensitivity, we examined a panel of tumor lines with activation of survival signaling at the level of EGFR or RAS (Fig. 1*D*). The SQ20B laryngeal squamous cell carcinoma line with EGFR overexpression showed a significant reduction in clonogenic survival after irradiation in the presence of PI-103, as did the T24 bladder cancer line expressing oncogenic H-RAS and the HT1080 sarcoma cell line expressing oncogenic N-RAS. To quantify the effect of the inhibitor, a comparison of the radiation dose required to reduce the surviving fraction to 10% was calculated for control and PI-103–treated cells. The ratio of the radiation dose for control cell to PI-103–treated cell to achieve this level of cell killing (dose modification factor or DMF_{10}) was calculated to be 1.9 for SQ20B, 1.7 for T24, and 1.4 for HT1080. Thus, there is significant radiosensitization of all three cell lines by this inhibitor.

Tumor cells with activated PI3K are radiosensitized by PI-103. We next examined whether activation of PI3K itself conferred resistance to PI-103 inhibition and to radiosensitization. Two colon cancer cell lines that express oncogenic K-RAS, DLD-1 and HCT-116, and derivatives of these lines that either expressed or did not express activated alleles of the PI3K p110α catalytic subunit (*PIK3CA*) were examined. Survival of all cells was reduced after PI-103 treatment (Fig. 2). Phosphorylation of AKT was present in all cells but was higher in cells expressing constitutively activated p110α (Fig. 3*A*). PI-103 treatment effectively inhibited AKT phosphorylation in all cells including both DLD-1 and HCT-116 with activated alleles of the *PIK3CA*. Thus, PI-103 was able to block AKT phosphorylation and reduce radiation survival in cells with activation of survival signaling at the level of K-RAS even if PI3K was also constitutively activated.

Effect of PI-103 treatment on immortalized and primary cells. To be useful as a radiosensitizing agent, PI-103 and inhibitors with a similar spectrum of inhibition would have to show a differential effect on tumor cell lines relative to normal cells. For this reason, we examined the radiation response of immortalized and primary cell cultures after exposure to PI-103. Treatment of these cells was done in the same way as for tumor cells with the same dose and timing of PI-103 exposure. AKT phosphorylation

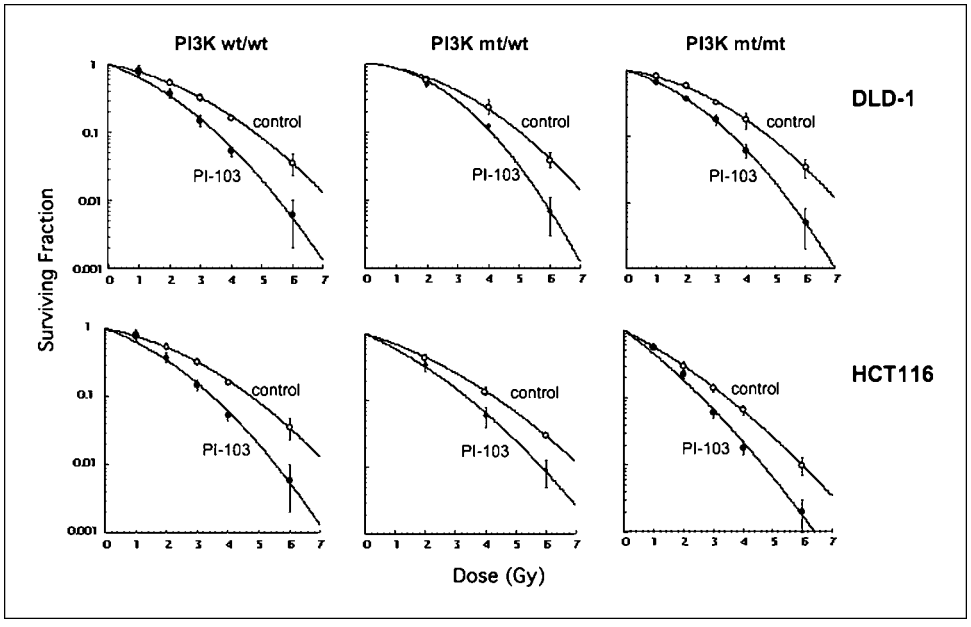

Figure 2. PI-103 reduces the radiation survival of cells expressing oncogenic K-RAS and constitutively active PI3K. Clonogenic survival of DLD-1 (*top*) and HCT116 (*bottom*). Log-phase cultures of cell lines of the indicated PI3K status were harvested and plated as single-cell suspensions. Cells were allowed to attach and were exposed to 0.4 μmol/L PI-103 (*closed symbols*) or DMSO (*open symbols*) for 1 h before irradiation at the doses indicated. After 14 to 21 d, plates were stained and scored for colony formation. *Points,* mean derived from at least three replicate plates; *bars,* SD. Plots are representative of replicate experiments.

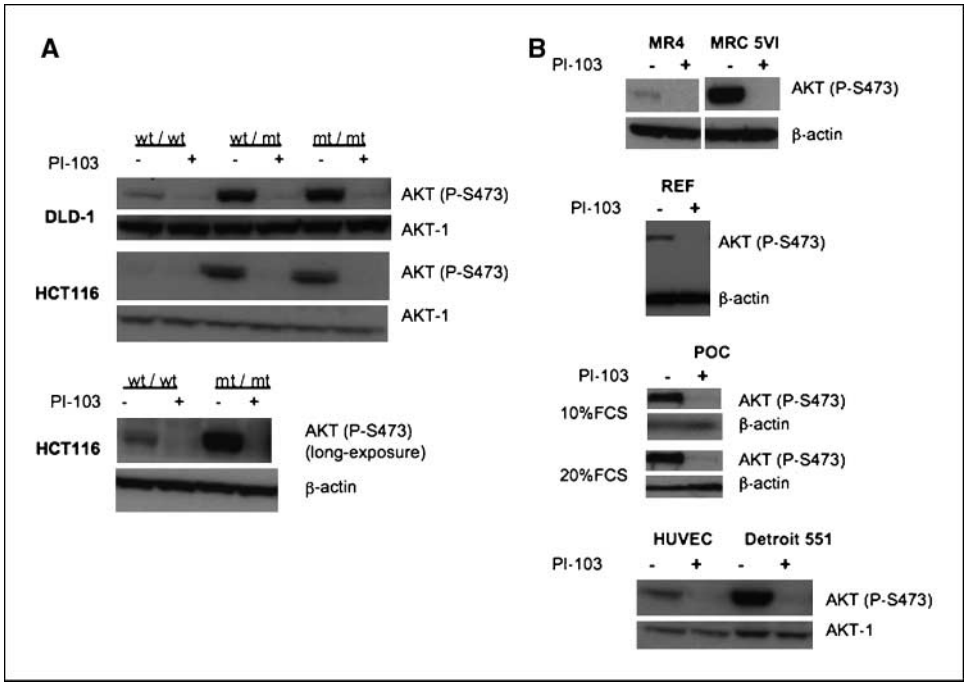

Figure 3. PI-103 reduces AKT phosphorylation in multiple cell types. *A,* AKT phosphorylation in cells expressing oncogenic K-RAS and constitutively active PI3K. Western blots show reduction in AKT phosphorylation on Serine 473 after PI-103 treatment in HCT116 and DLD-1 cell lines expressing wt PI3K (*wt/wt*) one allele (*mt/wt*) or two alleles (*mt/mt*) of constitutively active p110α at the time of irradiation for clonogenic survival (Fig. 2). Total AKT is shown as a loading control. A longer exposure is shown to demonstrate the presence of phospho-AKT in HCT116 wt/wt cells. *B,* AKT serine 473 phosphorylation in immortalized and primary cell cultures after PI-103 treatment. PI-103 treatment effects on AKT S-473 phosphorylation were determined at the time of irradiation for clonogenic survival (Fig. 4).

was monitored at the time of irradiation. Surprisingly, many of our "normal" cell cultures showed elevated AKT phosphorylation (Fig. 3*B*). The human fibroblast cells (MRC5, MRC5 VI, and POCp6) showed higher levels of phospho AKT than the two rat embryo fibroblasts–derived cells (MR4 and REFp10). Clonogenic survival determinations were carried out on both the immortalized cells (MRC5, MRC5 VI, and MR4) and on low passage cultures of primary cells (REF passage 10 and POC passage 6; Fig. 4). The effect of PI-103 treatment was minimal on the rat embryo cells, in which survival curves diverged significantly only at 6 Gy. The dose modification factor was 1.07 for REF and 1.18 for MR4 cells at 10% survival. The human fibroblasts showed both elevated AKT phosphorylation and greater radiosensitization than the rat embryo cells when irradiated in the presence of PI-103. This effect was not influenced by changing the serum concentration in the culture medium POC cells or testing different passage numbers (data not shown). The mean dose modification factor for POC cells was 1.4, and 1.31 for MRC5 VI cells. Similar results were obtained in MRC5 cells (data not shown). As a consequence of the activation of AKT in these cells, further clonogenic assays on other non-transformed cells were undertaken. Human umbilical vascular endothelial cell (HUVEC) cells showed no significant effect of PI-103 treatment on survival with a dose modification factor of 1.1 at 10% survival, whereas Detroit 551 cells showed a dose modification factor of 1.36 at 10% survival. The effect of PI-103 on survival in these two cell lines correlated with their respective levels of phospho AKT (Fig. 3*B*).

PI-103 sensitization is accompanied by increased G_2 delay and persistence of γH2AX foci. To determine whether radiosensitization was associated with inhibitor-mediated cell cycle redistribution, we compared the cell cycle distribution of SQ20B and T24 tumor cells, and REF and MRC5 fibroblasts after PI-103 treatment, and also examined the effects of this treatment on cell cycle delays after irradiation. None of the cell lines showed a significant effect of PI-103 treatment for up to 24 hours. However, if cells were irradiated with 4 Gy under PI-103 inhibition, the percentage of cells in G_2-M in T24, SQ20B was increased ~2-fold over the increase seen with radiation alone, whereas in MRC5 cells, it was increased by a third (Fig. 5). In contrast, REF cells, which are not radiosensitized, showed no accumulation in G_2-M after combined PI-103 and radiation. Similar, but less pronounced, effects were seen after two Gy irradiation (data not shown). The percentage of irradiated T24 and SQ20B cells treated with PI-103 that were in mitosis (1.2 ± 0.7 and 4.4 ± 1.1, respectively) was not significantly different from the percentage seen in irradiated T24 and SQ20B cells treated with carrier (1.8 ± 0.6 and 3.6 ± 0.8, respectively). Thus, the G_2-M arrest observed by flow cytometry was not due to increased accumulation in M.

The enhanced G_2 accumulation after irradiation in the presence of PI-103 indicated that there could be increased or persistent DNA damage in the PI-103–treated cells. We therefore examined the induction and resolution of DNA breaks by measuring the number of γH2AX foci at different times after irradiation. PI-103–treated T24 and SQ20B cells were compared with LY294002-treated cells. LY294002 has previously been shown to enhance radiation killing of these tumor cell lines and is a broad-specificity inhibitor of PI3K family members. Levels of γH2AX foci were comparable in cells irradiated in the presence or absence of PI-103 or LY294002 at 30 min (Supplementary Figs. S2 and S3) and 6 hours (data not shown) postradiation with 2 to 6 Gy. After 24 hours, a higher number of residual γH2AX foci were observed in tumor cells treated with either PI-103 or LY294002 compared with radiation alone. Similar results were obtained when Rad 51 foci were quantitated (Supplementary Fig. S3; Fig. 6*A* and *B*). In contrast to tumor cells, REF cells, which have low levels of phospho-AKT, showed a radiation dose-dependent increase in γH2AX foci but showed no difference in foci between either PI-103 or LY294002–treated cells and untreated cells at any radiation dose (Fig. 6*A*). Few Rad 51 foci were detected at 24 hours in any of the REF cell treatment groups. MRC5 cells showed an intermediate response with γH2AX foci being

elevated at 24 hours, consistent with their high level of AKT phosphorylation and enhanced sensitivity after PI-103 treatment in clonogenic survival tests, whereas Rad 51 foci levels were at unirradiated cell levels.

Levels of γH2AX detected by Western blot (Supplementary Fig. S2*B*) were elevated to an equivalent extent 30 min after irradiation in both control and PI-103–treated tumor cells. The levels of phospho-H2AX protein fell over the next 24 hours. No consistent alteration of total γH2AX after PI-103 treatment was seen. Thus, the persistence of foci in PI-103–treated cells was not a reflection of an overall increase of cellular γH2AX but rather a specific localization of this phosphorylated protein to foci. Together, these findings indicate that treatment with PI-103 does not enhance the induction of DNA damage but does result in the persistence of the damage induced by radiation in cells that are sensitized by treatment with PI-103. This persistent damage is consistent with the enhanced G_2 block seen in sensitized cells after irradiation in the presence of this inhibitor (Fig. 5).

Discussion

It has been well-documented that a subset of the signaling pathways activated in many tumors leads to enhanced survival after DNA damage caused by chemotherapy or radiotherapy (7, 9, 30, 31). Targeting these pathways has been a focus of recent research toward the development of monotherapies but may be an even more attractive target for enhancing the efficacy of existing cytotoxic modalities because some tumor cells could survive inhibition of survival pathways in the absence of concurrent cytotoxic treatment. One of the primary signaling hubs in survival signaling is PI3K. PI3K activity is elevated in many tumors as a result of signals originating at tyrosine kinase receptors or at RAS. Furthermore, activating mutations in PI3K itself have been identified in some tumors (32–34), and loss of PTEN, which down-regulates signaling from PI3K, is also common (35). PI3K activation has also been reported as a mechanism of Imatinib treatment resistance (36).

The range of inhibitors of PI3K that were until recently available was limited and included wortmannin, a fungal metabolite, and the synthetic LY294002 inhibitor. Although radiosensitization has been reported using both of these inhibitors, neither is specific for PI3K, and they show significant toxicities *in vivo* (23, 24). More recent work has led to the development of multiple inhibitors targeting PI3K activity, which are in varying phases of preclinical development (28, 29, 37, 38). Many of these newer inhibitors have greater specificity for particular PI3K isoforms (25, 39) and may thus show reduced *in vivo* toxicity.

PI-103 has been shown to be selective for class I PI3Ks but also has activity against mTOR and DNA-PK (26, 29). As a single agent, it has been shown to have activity against U87MG glioblastoma (29). PI-103 has also been shown to inhibit breast, and ovarian cancer xenograft growth, as well as the PC3 prostate and HCT-116 colon cancer cells used in our studies (26).

Indications for developing inhibitors of PI3K as radiation sensitizing agents. Our past work and that of others point to PI3K as a likely mediator of enhanced tumor survival after radiation-induced DNA damage. We have shown that inhibiting the expression of PI3K or its downstream target AKT using siRNA resulted in radiosensitization of several tumor cell lines including those presented in this report (18). The selective sensitization of tumor cells at the level of PI3K has the advantage that it blocks survival signaling originating at several points. As examples, EGFR and other tyrosine kinase receptors promote radiation survival signal through PI3K (40, 41). RAS signaling also activates PI3K (9). In addition, interaction of oncogenic RAS with PI3K seems to be required for transformation *in vivo* (42). Activation of PI3K itself promotes radiation survival (9), which we have shown here is susceptible to inhibition. PI3K and AKT signaling have also been implicated in promoting DNA repair through DNA-PK activation (43). The effect of AKT on radiation sensitivity was shown directly by showing that cells with constitutive activation at the level of AKT were not radiosensitized by LY294002 (44). Interestingly, the combination of LY294002 inhibition and radiation did not enhance the G_2 delay in the LNCaP tumor model used in that study. This could be attributable to the length of inhibitor treatment before

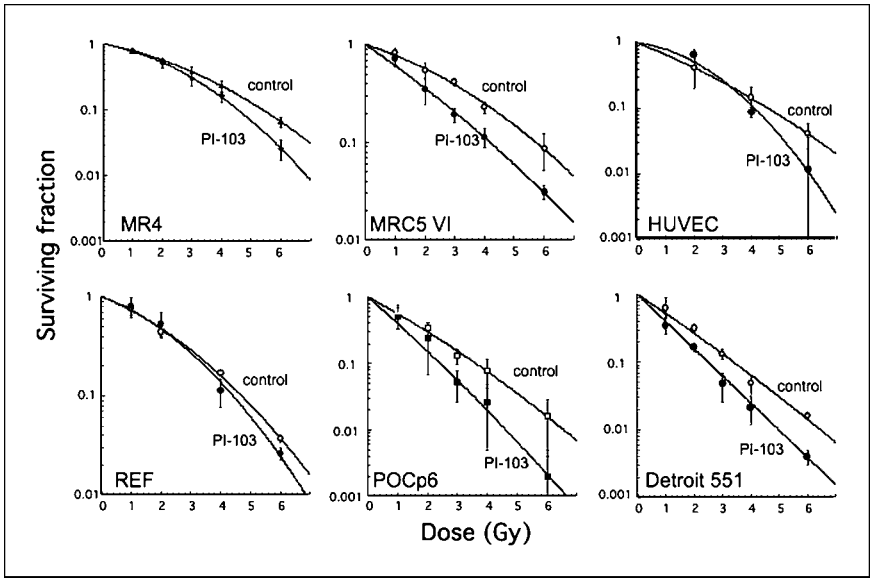

Figure 4. Clonogenic survival of immortalized and primary cell cultures after PI-103 treatment. Clonogenic survival of MR4 (v-myc–immortalized rat fibroblast), MRC5VI (lgT-antigen–immortalized human lung fibroblast), REF (short-term primary rat fibroblast), POC (passage 5 primary cultures of human fibroblasts), HUVEC, and Detroit 551 (human fibroblasts cells) was measured after 0.4 μmol/L PI-103 treatment (*filled symbols*). *Points,* mean derived from at least three replicate plates; *bars,* SD. Plots are representative of replicate experiments.

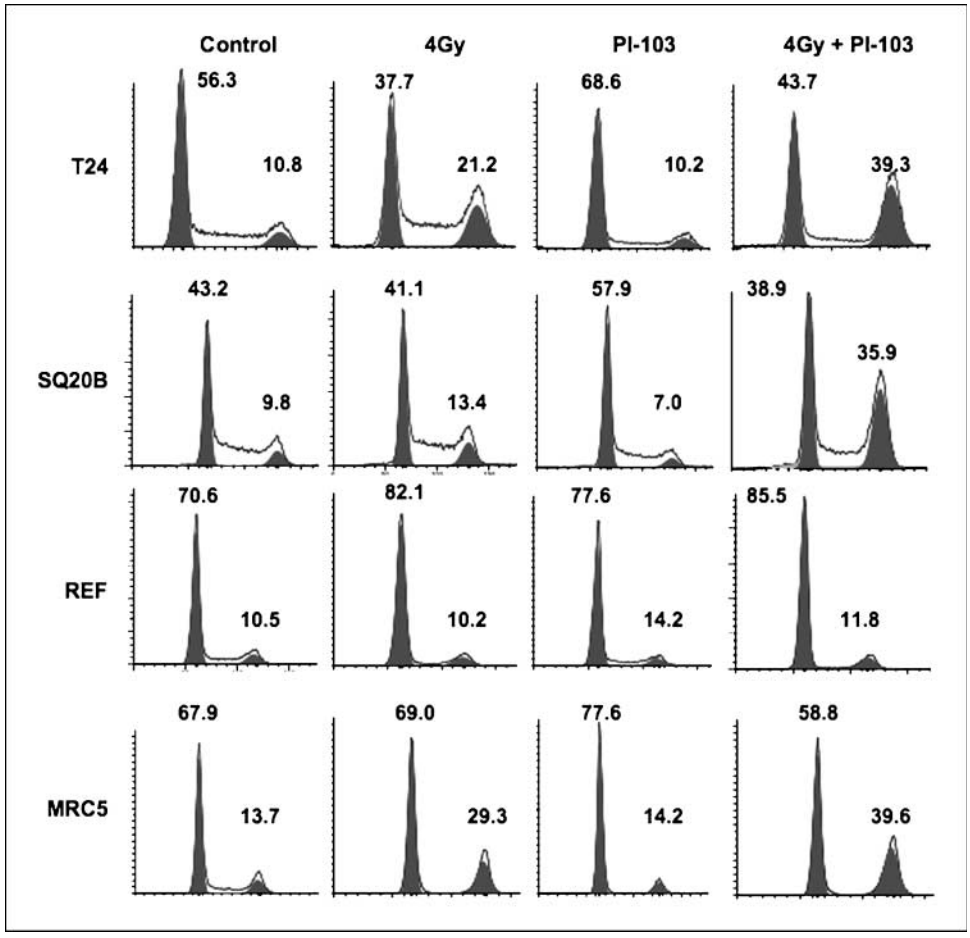

Figure 5. Cell cycle distribution after PI-103 treatment, irradiation, or combined treatment. Cells were treated with 0.4 μmol/L PI-103 and irradiated with 4 Gy. Samples were harvested for flow cytometry at 24 h. Numbers above G_1 and G_2-M peaks indicate the percentage of cells in each compartment.

irradiation, 24 hours rather than the 1 hour used here, or the difference in cell model. Another radiosensitization strategy using PI3K inhibition has been adopted by Hallahan and colleagues (50). This group has targeted tumor vascular endothelial cell survival using p110δ–specific inhibitors with significant effects on tumor regrowth. Thus targeting PI3K may be applicable to a wide range of tumors to enhance radiosensitivity either by targeting the tumor cell or the tumor vasculature.

PI-103 is a potent radiation sensitizer for human tumor cells. The results we present here show that PI-103 is a potent sensitizer of tumor cells with activation of signaling originating at the EGFR, or due to oncogenic mutation in H-, K-, or N-*ras*. We further showed that PI-103 can inhibit phosphorylation of AKT in cells with constitutively active PI3K. Thus, the efficacy of this compound in inhibiting PI3K applies to tumor cells with a wide spectrum of oncogenic lesions. Results with PC-3 cells that are PTEN mutant indicate that cells with this lesion are also very susceptible to inhibition, although we were not successful in testing PC-3 for radiosensitization due to the single agent toxicity of PI-103 in this cell line. Sensitization to radiation seems to result from the persistence of DNA damage in PI-103–treated cells as evidenced by elevated levels of γH2AX and Rad 51 foci 24 hours postirradiation. Both γH2AX foci, resulting from phosphorylation of the histone H2AX at Ser 139 by DNA-PK, ATM and other kinases (46), and Rad 51 foci are markers for sites of DNA double strand breaks. These breaks can include both those produced promptly in S-phase and G_2-M as a result of radiation as well as those formed in the process of DNA replication. γH2AX is thought to act in the recruitment of DNA repair complex proteins to sites of double-strand breaks, whereas Rad 51 is an integral part of the homologous recombination machinery (47). Our observation of persistent foci after PI-103 and LY294002 treatment is in accord with the results of Toulany and colleagues (40) who showed decreased survival and persistence of foci in lung cancer cells after irradiation in the presence of an EGFR inhibitor. Our results also concur with recently published findings of Kao and colleagues (48) who showed that treatment with LY294002 or inducing PTEN expression in glioblastoma cells led to radiosensitization and γH2AX persistence. As in the latter report, we did not observe a consistent reduction in phospho-H2AX after PI3K inhibition as assessed by Western blotting of total cellular protein. This finding implies that PI-103 exposure does not interfere with phosphorylation of H2AX after DNA damage.

The specificity of PI-103 inhibition. The demonstration by Knight and colleagues (25) showing that the specificity of PI-103 in enzymatic assays of inhibition is greater for DNA-PK than for PI3K p110α has caused us to carefully examine the effects of PI-103 on normal cells because inhibition of DNA-PK in normal cells could lead to the persistence of some double-strand breaks and enhance normal tissue responses to radiation-induced DNA damage by retarding nonhomologous end joining. Our initial studies showed low levels of AKT phosphorylation and no radiosensitization of the POC human fibroblasts (data not

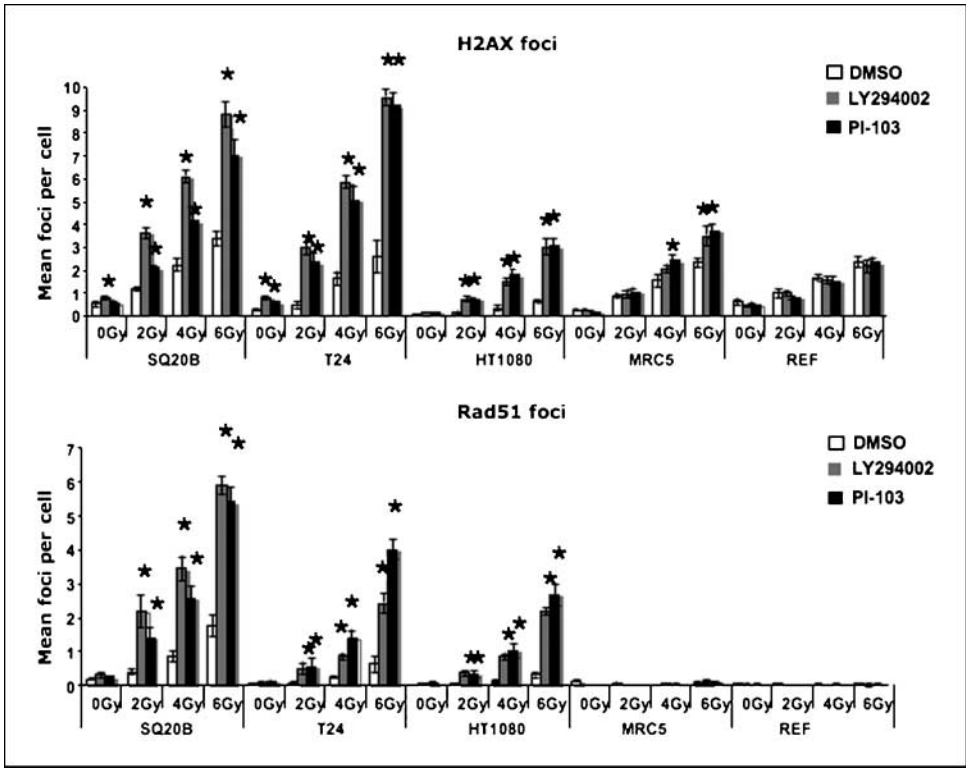

Figure 6. Evaluation of residual DNA damage (γH2AX and Rad 51 foci) after PI-103 treatment and irradiation. Residual DNA damage was quantitated by counting γH2AX (*top*) and Rad 51 (*bottom*) foci in cells irradiated with 4 Gy after 1-h treatment with PI-103 (0.4 μmol/L), LY294002 (10 μmol/L), or DMSO carrier (controls). Cells were fixed 24 h after irradiation and stained for γH2AX or Rad 51 as described in Materials and Methods. Images were acquired on a GE IN Cell Analyzer, and values were obtained after analysis on IN Cell Analyzer Workstation software. Two-tailed *t* tests were used to determine the significance of the observed increases in foci between irradiated control and irradiated inhibitor-treated cells at each radiation dose; *, $P < 0.01$.

shown), but over time, these cells have acquired elevated AKT phosphorylation and become susceptible to sensitization by PI-103. Our recent results indicate that the degree of radiosensitization seen in untransformed cells correlates well with the level of activating AKT Ser 473 phosphorylation. Some immortalized and primary cells were susceptible to sensitization in proportion to the levels of AKT phosphorylation they displayed. REF cells, with the lowest levels of phospho-AKT, showed no significant effect of PI-103 on radiosensitivity as measured either by clonogenic survival or as assessed by quantitating residual γH2AX or Rad 51 foci. MR4 cells with low levels of phospho-AKT also showed little radiosensitization. Other immortalized or primary cell lines showed sensitization to the extent that they exhibited AKT activation (phosphorylation at Ser 473). HUVEC cells showed less sensitization than Detroit 551, which had elevated AKT phosphorylation, whereas MRC5 and MRC5 VI, with high levels of phospho-AKT, showed sensitization by PI-103 that is equivalent to that of tumor cells. It is possible that the elevation of phospho-AKT is an artifact of *in vitro* culture. Because most normal human cells show low levels of AKT phosphorylation *in situ* (49), we would predict that the sensitizing effect of inhibitors like PI-103 will be modest for normal cells relative to the sensitization of tumors. PI-103 did sensitize all tumor cells tested, the majority of which show high levels of AKT phosphorylation. Our past studies and those of other groups have implicated AKT as a mediator of radiation survival (1, 3, 18, 31, 50, 51). Although the response to PI-103 in DLD-1 and HCT116 cells and the derivative lines expressing mutant PI3Kinase did not correlate with AKT activation levels, the inhibition achieved by PI-103 treatment was sufficient to block AKT phosphorylation in all the cells irrespective of the initial level. In both DLD and HCT116, the radiation survival after PI-103 treatment was equivalent irrespective of PI3K status, demonstrating that the activation of PI3K itself was insufficient to render these cells resistant to the inhibitor.

The demonstration by Fan and colleagues (29) that PI-103 also inhibits mTOR could also effect on its effects *in vivo*. Inhibition of mTOR could cause altered responses to irradiation in the tumor and host vasculature (52). Enhancing damage to tumor vasculature could promote tumor killing, whereas in normal host vasculature, it could potentiate radiation injury. The possible effects of PI-103 on normal tissue radiation responses will need to be examined in further tests *in vivo*.

Although our *in vitro* results indicate that inhibitors such as PI-103 show a therapeutic index and may be applicable in the context of radiation therapy, newer generation PI3K inhibitors with greater specificity for PI3K isoforms are being developed and may be advantageous in the context of radiotherapy. Our results show that this approach may have wide applicability in the context of radiation therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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