Phosphatidylinositol 3-Kinase Inhibition by LY294002 Radiosensitizes Human Cervical Cancer Cell Lines

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Radiation therapy plays a pivotal role in the treatment of advanced-stage cervical cancer. However, roughly 40% to 95% of women diagnosed with advanced-stage cervical cancer will succumb to their disease (1–3). Intracellular communication pathways leading to promotion of tumor growth, mitotic cell death, or apoptosis provide promising targets of intervention in the complex process of radiosensitization.

Abstract

Purpose: The phosphatidylinositol 3-kinase (PI3K) catalytic subunit is amplified in cervical cancers, implicating PI3K in cervical carcinogenesis. We evaluated the radiosensitizing effect of PI3K inhibition by LY294002 on clonogenic survival, growth characteristics, and gene expression in cervical cancer cell lines (HeLa and CaSki).

Experimental Design: Cervical cancer cells were treated separately and concurrently with the PI3K inhibitor LY294002 (10 μmol/L) and radiation (2 Gy) with serial analysis of cell count, apoptosis, and flow cytometry. PI3K inhibition was assessed by protein analysis of phosphorylated Akt. Clonogenic assays were done with varying doses of radiation and LY294002 and varied time points of administration of LY294002 proximate to the radiation dose. Surviving fractions and dose modification factors (DMF) were calculated. Each experiment was done in triplicate and analyzed using ANOVA regression analysis and Dunnett’s t Test. Microarray gene expression analysis was done on the HeLa cell line.

Results: PI3K inhibition with LY294002 alone did not decrease cell survival. However, treatment with LY294002 significantly radiosensitized HeLa and CaSki cell lines with DMFs (1 log cell kill) of 1.95 and 1.37, respectively. Compared with post-irradiation, pretreatment produced more radiosensitization (P < 0.0001). DMFs were 2.2, 2.0, 2.0, and 1.2 for LY294002 added at 0, 6, 2, and 0.5 hours before irradiation and 6 hours after irradiation, respectively. LY294002 pretreatment in irradiated HeLa cells led to altered gene expression.

Conclusions: Although LY294002 alone did not produce cytotoxic effects, PI3K inhibition with LY294002 produced significant radiosensitization, showed significant time-dependent effects, increased apoptosis, and altered gene expression. These findings support future investigation of PI3K inhibitors in combination with radiation therapy for carcinoma of the cervix.

The phosphatidylinositol 3-kinase (PI3K) family of enzymes is well characterized with respect to promotion of cellular growth, survival, and suppression of apoptosis in cancer cells (4–6). These kinases can be activated from a cell surface growth factor receptor (such as epidermal growth factor receptor) and are known to play a critical role in regulating the balance between cell survival and apoptosis. PI3K enzymes are cytosolic and consist of both regulatory and catalytic subunits, which regulate a vast array of fundamental cellular responses (7).

Increased signaling through this pathway has been shown to lead to downstream phosphorylation of Akt in vitro and in vivo (8). Increased intratumoral phosphorylated Akt has been linked to decreased radiation responsiveness in various malignancies, including head and neck squamous cell carcinoma, lung carcinoma, glioblastoma, and prostate and breast cancers (9–13). Thus, inhibition of PI3K, or molecules involved in the PI3K signaling pathway may provide a directed approach to therapy for this disease process.

Among gynecologic cancers, a role for PI3K in cervical carcinogenesis has been recently suggested. Observations that amplification in 3q is the most frequent chromosomal aberration noted in cervical cancer and that the gene encoding the p110α subunit (PIK3CA) resides at chromosome 3q26 has prompted this line of investigation. PIK3CA has been shown to...
be amplified in tumor samples of cervical carcinomas as well as in cervical carcinoma cell lines (SiHa, C33a, and ME-180; refs. 14, 15). In other gynecologic malignancies, PIK3CA has thus far only been implicated in ovarian carcinogenesis (16).

PI3K inhibition by 2-(4-morpholinyl)-8-phenyl-chromone (LY294002) has been shown to cause apoptosis in various human cancer cells in vitro (17–20). An inhibitor of all major subclasses of PI3K and PI3K-like kinases (ATM, ATR, and DNA-PK), LY294002 has been evaluated in various cell lines showing increased apoptosis (19, 20). Few in vivo studies have been done assessing the effects of LY294002 in tumor control (21–23). Two of these studies administered the LY294002 alone and in combination with paclitaxel in an ovarian carcinomatosis mouse model and found significant inhibition of tumor growth and ascites formation (21, 22). As a radiosensitizer, LY294002 has shown improved radiation efficacy in a bladder cancer cell xenograft model (23).

In this study, we examined the effects of PI3K inhibition by LY294002 in human cervical cancer cell lines exposed to ionizing radiation. Our aims were to assess changes in radiosensitivity, cell growth characteristics, and patterns of gene expression with the addition of LY294002 concurrent with radiation therapy. Additionally, we sought to evaluate how permutations in the timing of LY294002 administration relative to the time of irradiation subsequently affect radiosensitivity.

Materials and Methods

Cells. HeLa cells were kindly provided by Dr. Ruey Min Lee (Huntsman Cancer Institute, Salt Lake City, UT). CaSkis were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were maintained in log growth phase in stock plates at 37°C in humidified air with 5% CO2. HeLa and CaSkis were cultured in DMEM and RPMI 1640, respectively. Media for all cells were supplemented with 10% fetal bovine serum. All cell culture reagents were obtained from Invitrogen Co. (Carlsbad, CA).

Cell growth curves, apoptosis, and flow cytometry. Cell lines were plated separately in 100-mm tissue culture dishes. Forty-eight hours after cells were seeded, the medium was removed and replaced with culture medium containing either the PI3K inhibitor LY294002 (10 μmol/L dissolved in DMSO) or DMSO only (Sigma Chemical, St. Louis, MO). The concentration of DMSO in both control and test groups did not exceed 0.5%. The medium containing LY294002 (10 μmol/L dissolved in DMSO) or DMSO alone was changed every 24 hours. For those plates receiving concurrent PI3K inhibition and radiation treatment, LY294002 was added to the cell cultures ≤1 hour before treatment with radiation. Cells were irradiated with a MARKI Cesium-137 Gamma Irradiator with 2 Gy at a dose rate of 4.35 Gy/min. Following irradiation, cells were collected at 0-, 24-, 48-, and 72-hour time points. At each time point, cell numbers were quantitated using a Beckman Coulter Counter and evaluated for apoptosis and cell cycle distribution.

Apoptosis was quantitated by CaspACE assay after preliminary studies showed improved sensitivity of CaspACE assay over Annexin V staining in HeLa cells (data not shown). Treated and control cells collected at each time point were washed twice with 1× PBS and stained with 10 μmol/L CaspACE FITC-VAD-FMK In Situ Marker (Promega Co., Madison, WI) at a density of 2×10^6/ml for 20 minutes. The cells were then centrifuged, the supernatant removed, and the cells resuspended in 1× PBS. The percentage of cells undergoing apoptosis was determined by flow cytometry (BD FAC Scan).

Separate plates of treated and control cells were collected at each time point, washed twice with 1× PBS, and stained with 10 μg/ml propidium iodide (Molecular Probes, Inc., Eugene, OR). Cell cycle distribution was determined by flow cytometry, and analysis was done with FACSComp 4.1 software. All experiments were done in triplicate.

Western blotting and PI3K activity inhibition by LY294002. Confirmation of inhibition of PI3K activity was done by Western blot analysis of one of its downstream targets, Akt. Following 48 hours of exposure to LY294002 at increasing concentrations, whole-cell protein extracts were prepared and analyzed by Western immunoblotting using the PhosphoPlus Akt (Ser473) Antibody kit (Cell Signaling Technology, Inc., Beverly, MA). The cells were lysed in a SDS sample buffer containing reduced and non-reduced sample buffer, sonicated briefly. Samples were boiled, normalized, and clarified by centrifugation and stored at −20°C. Samples containing equal amounts of protein were separated on a SDS-PAGE gel and blotted onto nitrocellulose membrane. Samples were incubated in blocking buffer before primary antibody addition. The membrane was probed first with a polyclonal anti-phospho-Ser-473 Akt (1:1,000 dilution) and then reprobed with horseradish peroxidase–conjugated secondary antibody (1:2,000 dilution) and horseradish peroxidase–conjugated anti-biotin antibody (1:1,000 dilution). The membrane was then reprobed with a polyclonal anti-pan Akt as a loading control. Antibody binding was detected using the Phototope-HRP Western Detection kit.

Determination of cell survival. The sensitivity of cells to radiation was measured using clonogenic assays. To measure clonogenic survival, exponentially growing cells were trypsinized, plated at known concentrations, and irradiated with a range of doses (0-10 Gy). Clonogenic survival experiments were done with varying concentrations of LY294002 (0, 5, 10, and 25 μmol/L) added 30 minutes before irradiation. Cells were grown for 9 to 12 days in media containing either LY294002 in DMSO or in media with DMSO alone. Cells were then fixed with methanol and stained with methylene blue. Colonies containing >50 cells were evaluated by light microscopy and scored as survivors. In each case, the survival fraction was calculated by dividing the number of colonies counted by the number of cells plated times the plating efficiency. Triplicate experiments were done for each cell line.

Schedule-dependent growth and clonogenic assays. Each growth characteristic assay (cell count, apoptosis, and cell cycle distribution) was done on cells collected at 48 hours following time of irradiation. The dose of 25 μmol/L LY294002 was selected as the optimal radiosensitizing dose based on results from the clonogenic assays. The studied time points of 25 μmol/L LY294002 addition proximate to the time of irradiation (2 Gy radiation administered at time 0 for all experiments) included 6 hours before, 2 hours before, 0.5 hour before, and 6 hours following. Control arms with no added LY294002 or irradiation and 2 Gy irradiation alone were also studied for comparison. Forty-eight hours following irradiation, cell number, apoptosis, and cell cycle distribution were determined as described above. All experiments were done in triplicate.

Clonogenic survival experiments were done with 25 μmol/L LY294002 added at various time points around the time of irradiation. LY294002 was present in the media until the first media change at 72 hours following irradiation. The studied time points of LY294002 administration with respect to the time of irradiation (time 0 defined as time of radiation administration) included 6 days before, 3 days before, 1 day before, 6 hours before, 2 hours before, 0.5 hour before, and 6 hours following. A control arm with no added LY294002 was also studied. Following irradiation, cells were grown for 9 to 12 days. Colony count and surviving fraction were determined as described above.

Microarray analysis of gene expression. Total RNA was extracted from each HeLa sample via an RNeasy Midi kit (Qiagen, Valencia, CA). Next, cDNA was synthesized by use of First Strand cDNA Synthesis kit K1612 (MBI Fermentas, Vilnius, Lithuania) from polyadenylate mRNA. The Microarray Core Facility at the University of Utah/Huntsman Cancer Institute consists of an Amersham Biosciences GEN III Array Spotter and a Gen III Array Scanner. To print microarrays, cDNA clones are PCR amplified and deposited onto a chemically modified glass surface by the Microarray Core Facility to form a high-density microscopic array of these genes. Hybridization of labeled RNA samples from pretreated...
cells versus untreated control cells to the microarray defines the genetic expression differences between the two states. The expression of 19,200 genes was analyzed in triplicate for each treatment condition versus control.

**Statistical analyses.** The clonogenic survival data were fitted using the linear quadratic model surviving fraction = \( \exp[-(\alpha D + \beta D^2)] \), where \( \alpha \) = initial slope and \( \beta \) = terminal slope of the survival curve. Dose modification factors were calculated from the fitted survival curves at the 1 log cell kill level using Prism GraphPad software. The surviving fraction was analyzed using the general linear modeling procedure of the statistical analysis software system. The mathematical model included the effects of treatment and dose of radiation and interaction of treatment with dose. In addition, tests for homogeneity of regression were also done by comparing the reduction of error sums of squares by fitting a curve for each treatment rather than using a single regression to describe all treatments. For cell growth experiments, rates of apoptosis were also determined by comparing the reduction of error sums of squares by fitting a curve for each treatment rather than using a single regression to describe all treatments. For cell growth experiments, rates of apoptosis and total cell count were evaluated between treatments by ANOVA at each time point tested to compare treatment differences with control.

The microarray study design consisted of triplicate experiments for each of the three treatment conditions and control. First, we formed a ratio of the expression value with the background of each of 19,200 genes represented. We considered only the genes with interpretable pairs of spots in all three replications. We then determined the mean and SD of this expression ratio for each array, across all genes analyzed. We produced a "standardized array" by subtracting the mean and dividing by the SD of each ratio. Under the assumption that the ratios are approximately normally distributed for each microarray, this standardized array should represent an approximate large sample from a standard normal distribution. Quantile-quantile plots of the data were then constructed for analysis. From the standardized arrays, a list of up-regulated and down-regulated genes (relative to control) was generated whose standardized ratio values were >2.0 or less than −2.0, which correlates with a \( z \) score of >5.0 or less than −5.0. Under normal distribution assumption, a \( z \) score of >5.0 will be significant at the 0.01 significance level (using a Bonferroni inequality).

**Results**

**PI3K inhibition by LY294002.** PI3K inhibition by LY294002 was seen in all cell lines tested, as shown by decreased phosphorylation of the downstream target, Akt. Sequential increases in LY294002 concentration led to decreased phosphorylation of Akt, as shown in Fig. 1, for the HeLa and CaSki cell lines. Once controlled for loading differences by quantifying using NIH imaging software and normalizing against the unphosphorylated Akt present, a clear dose-response is seen with increasing LY294002 concentrations.

**Cell growth characteristics.** Figure 2 illustrates the cell growth of HeLa and CaSki cells, respectively, expressed as a percentage of the cell growth obtained by the control population at various time points. Consistent with previous reports (24), CaSki cells were relatively more radioresistant than HeLa cells. Cell counts of the irradiated CaSki cells were equal to 86% of untreated cell population at 72 hours after irradiation. In contrast, by 48 hours, irradiated HeLa cells achieved only 70% of the growth measured in the nonirradiated cells. Despite this radioresistance, the addition of LY294002 in CaSki cells resulted in significant growth restriction at 72 hours, when exposed either to the inhibitor alone or in combination with radiation (\( P < 0.001 \)). Although PI3K inhibition and irradiation both seem to decrease cell growth individually and in combination in both HeLa and CaSki cells, only the 72-hour CaSki cells showed a statistically significant difference.

A significant decrease in the percentage of viable cells was seen in the combination radiation/LY294002 treatment group (72.2%) compared with control, radiation, or LY294002-treated cells (97.2%, 94.1%, 91.4%, respectively). The decrease in viable cells in the combination group corresponded to a significant increase in both early apoptotic (15.0%) and dead (12.8%) cells (see Fig. 3). No significant alterations in cell cycle distribution were seen in any treatment group, at any time point tested.

**Radiosensitization by LY294002 measured by clonogenic assay.** Clonogenic potential was assessed by colony formation assays following administration of LY294002 at increasing concentrations, alone and in combination with increasing doses of radiation. Figure 4 illustrates the HeLa clonogenic assay results. Figure 4A shows the surviving fraction of clones exposed to varied concentrations of LY294002 (no radiation was administered during this experiment). At all concentrations tested (0-25 \( \text{mmol/L} \)), LY294002 alone did not alter cell survival, indicating that replicative potential was not impaired at these doses of the PI3K inhibitor. However, increasing concentrations

![Fig. 1. PI3K inhibition by LY294002.](https://www.aacrjournals.org/doi/figure/10.1158/1078-0432.CCR-05-1660)

**A**, dose-dependent inhibition of PI3K by LY294002 shown by increasing suppression of Akt phosphorylation. **B**, dose-dependent PI3K inhibition expressed as the percentage of phosphorylated Akt (p-Akt) relative to untreated cells (controlled for loading differences by normalization of total Akt present). Medium containing DMSO was used as control for both cell lines when no LY294002 (0 \( \text{mmol/L} \)) was added to the medium.
of LY294002 yielded significant radiosensitization across 0 to 10 Gy, with $P < 0.0001$ for both HeLa and CaSki cells (Fig. 4B and C). Treatment with 25 μmol/L LY294002 showed significant radiosensitization of the HeLa and CaSki cell line with a dose modification factor of 1.95 and 1.37, respectively, for 1 log cell kill. Differences in both the initial ($\alpha$) and terminal ($\beta$) slopes of the survival curves were found between the combination 25 μmol/L LY294002 and radiation-treated cells compared with radiation-only–treated HeLa cells (see Table 1). The changes in both $\alpha$ and $\beta$ slopes at the 25 μmol/L concentration indicate a synergistic, or supra-additive, effect of LY294002 on the radiosensitivity of HeLa cells.

**Schedule-dependent growth characteristics and radiosensitization.** At 6, 2, 0.5 hours pretreatment and 6 hours post-treatment experiments, analysis of cell count at 48 hours following radiation, with and without LY294002, showed a significant decrease in cell numbers for both LY294002 and radiation/LY294002-treated cells compared with untreated

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Fig. 2. Cell growth assay. Cells treated with 10 μmol/L LY294002, 2 Gy, and in combination. Cell numbers were counted at 0, 24, 48, and 72 hours following radiation. A, HeLa cells. B, CaSki cells.

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Fig. 3. Apoptosis assay. Percentage of viable, early apoptotic, and dead cells present following treatment of HeLa cells with radiation, LY294002, and both in combination. Medium containing DMSO was used as control when no LY294002 (0 μmol/L) was added to the medium.

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Fig. 4. Clonogenic survival. A, HeLa cells treated with LY294002 alone. B, HeLa cells treated with increasing doses of LY294002 and radiation. C, CaSki cells treated with increasing doses of LY294002 and radiation.
controls ($P < 0.0001$). However, no synergism was noted between LY294002 and radiation at these doses. No effect of LY294002 on levels of apoptosis was seen, and no interaction between LY294002 and radiation was detected. Radiation-treated cells (with and without LY294002) showed a significant ($P = 0.017$) increase in $G_1$ and a trend towards a decrease in $G_2$ distribution compared with nonradiated cells ($P = 0.0866$). No significant interaction was noted between LY294002 and radiation with respect to cell cycle distribution (data not shown).

Figure 5 illustrates clonogenic assay results for 0 to 10 Gy irradiation with varied timing of administration of 25 $\mu$mol/L LY294002. The data shown are the means ± SE of three independent experiments. Pretreatment with LY294002 at all time points tested yielded significant radiosensitization across 0 to 10 Gy ($P < 0.0001$). In contrast, initiation of PI3K inhibition 6 hours after irradiation produced minimal but statistically significant radiosensitization. No difference was seen between the 0.5-, 2-, and 6-hour pretreatment groups. Compared with post-irradiation treatment, pretreatment produced significantly more radiosensitization ($P < 0.0001$). Surviving fraction at 2 Gy (SF2) values were significantly lower in the pre-irradiation-treated cells only compared with control (SF2 = 0.76, 0.33, 0.42, 0.42, for control; 6, 2, 0.5 hours pretreatment, respectively; $P < 0.0001$). SF2 for cells treated 6 hours after irradiation was not different from radiation-only control cells (SF2 = 0.76 versus 0.89, control versus 6 hours after irradiation; $P = 0.20$). The dose modification factors for 1 log cell kill were 2.2, 2.0, 2.0, and 1.2 for LY294002 added at 6, 2, 0.5 hours pretreatment and 6 hours posttreatment experiments, respectively.

**Microarray analysis.** The HeLa cell line was studied with microarray analysis. Of the 19,200 genes spotted onto the arrays, ~11,000 to 1,200 genes (variable for each triplicate experiment) were interpretable across all three experiments and therefore deemed acceptable for analysis. Those genes found to be significantly up-regulated or down-regulated at a significance level of $P < 0.01$ are listed in Table 1. Of note, four genes, which were significantly up-regulated by radiation, were not up-regulated in the combination LY294002/radiation group [androgen receptor associated protein 24, muscarinic acetylcholine receptor M3, heterogeneous nuclear ribonucleoprotein A2/B1, and ELK4]. Although the ribosomal protein L9 was consistently down-regulated in the LY294002, radiation, and LY294002/radiation groups, other genes were found to be down-regulated by either treatment alone but not in the combination group (cell matrix adhesion regulator variant and ribosomal protein S3A; see Table 2).

### Discussion

Recently, PI3K, its upstream signals, and downstream targets have generated much interest as mediators of radiation resistance. Studies of PI3K inhibition in other human cancer cell lines have shown that the nonselective PI3K inhibitors LY294002 and wortmannin have radiosensitizing effects (3). The majority of these studies have evaluated nongynecologic carcinomas, the current study being the first to evaluate both synergistic effects of LY294002 and radiation and the cell growth characteristics of cell cycle distribution and apoptosis. This study has shown significant radiosensitization by the PI3K

### Table 1. Comparison of $\alpha$ and $\beta$ components of survival curves with or without LY294002

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$, Gy$^{-1}$ (95% confidence interval)</th>
<th>$\beta$, Gy$^{-2}$ (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 $\mu$mol/L LY294002 and radiation</td>
<td>0.19 (0.10-0.28)</td>
<td>0.20 (0.005-0.040)</td>
</tr>
<tr>
<td>25 $\mu$mol/L LY294002 and radiation</td>
<td>0.06 (0.037-0.087)</td>
<td>0.17 (0.15-0.18)</td>
</tr>
<tr>
<td>CaSki</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 $\mu$mol/L LY294002 and radiation</td>
<td>0.58 (0.50-0.65)</td>
<td>0.002 (−0.02 to 0.03)</td>
</tr>
<tr>
<td>25 $\mu$mol/L LY294002 and radiation</td>
<td>0.76 (0.41-1.10)</td>
<td>0.016 (−0.13 to 0.16)</td>
</tr>
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Fig. 5. Clonogenic survival. A, HeLa cells treated with radiation and 25 $\mu$mol/L LY294002 added at varied time points proximate to the radiation dose (6 hours before, 2 hours before, 0.5 hour before, and 6 hours after irradiation). B, extended pre-irradiation exposure (25 $\mu$mol/L LY294002 added 6 days before, 3 days before, and 1 day before irradiation). Once added, LY294002 exposure continued until completion of the experiment.
Table 2. Increased and decreased expression of genes with addition of LY294002 and radiation

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Abbreviation</th>
<th>z score</th>
</tr>
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<tbody>
<tr>
<td>Lecithin cholesteryl acyltransferase</td>
<td>LCAT</td>
<td>-5.82</td>
</tr>
<tr>
<td>Human Aac11 (aac11) mRNA, complete cds</td>
<td>PABPL1</td>
<td>-6.85</td>
</tr>
<tr>
<td>Human mRNA for KIAA0088 gene, partial cds</td>
<td>GAPD</td>
<td>5.80</td>
</tr>
<tr>
<td>Ribosomal protein L9</td>
<td>RPL9</td>
<td>-8.85</td>
</tr>
<tr>
<td>H. sapiens cell matrix adhesion regulator variant mRNA</td>
<td>RPS3A</td>
<td>-11.66</td>
</tr>
<tr>
<td>Six separate gene sites, ESTs</td>
<td>RPL31</td>
<td>-6.43</td>
</tr>
<tr>
<td>Increased expression for LY294002/XRT vs control</td>
<td>RPL12</td>
<td>-7.04</td>
</tr>
<tr>
<td>Ribosomal protein L9</td>
<td>RPL9</td>
<td>-7.09</td>
</tr>
<tr>
<td>Human mRNA for KIAA0088 gene, partial cds</td>
<td>RPL3</td>
<td>-9.08</td>
</tr>
</tbody>
</table>

In our study, radiosensitization was accompanied by increased levels of apoptosis. The activated Ras/PI3K/Akt pathway, a prime suspect in the radioresistant phenotype, has been elegantly shown to lead to direct inhibition of proapoptotic proteins and regulate transcription of proapoptotic and antiapoptotic genes (25). Numerous studies have implicated the Ras/PI3K/Akt pathway in radioresistance both in vivo and in vitro (3, 13, 23, 26–28). It is particularly interesting that PI3K inhibition alone did not significantly increase apoptosis. Rather, only the combination of radiation and LY294002 led to increased apoptosis and cell death. Recent evidence, however, has shown that radiation and chemotherapy-induced cell death in solid tumors is not always correlated with apoptosis (24, 29–31), suggesting that cell death via mitotic catastrophe and terminal growth arrest (senescence) may have larger roles than previously suspected.

Lastly, another possible candidate pathway mediating radiosensitization by LY294002 is that of ATM, ATR, and DNA-PK inhibition. ATM and ATR, which function in all known cell cycle checkpoints, and DNA-PK, which oversees DNA double-strand break repair via nonhomologous end-joining pathway, serve pivotal roles in DNA damage signaling and repair (32). Although the radiosensitizing effects of loss of G1 arrest vary across cell lines, G2 arrest seems to improve survival of cells after irradiation (33). Inhibitors of ATM and ATR, such as the methylxanthine caffeine, abrogate the G2 delay and have increased radiosensitivity (33). Inhibition of DNA-PK by LY294002 may have also contributed to the radiosensitization of cervical cancer cells in this study via its role in double-strand break repair. As noted by Rosenzweig et al. (34), DNA-PK inhibition by either wortmannin or LY294002 was seen in cells radiosensitized with these inhibitors, with increase in 75% of cells in G2 at 50 hours after irradiation. Cells deficient in DNA-PK, however, were also partially sensitized to radiation by the inhibitors.

The known actions of the PI3K family and of PI3K inhibition have been elaborated by many investigators (7, 25, 26). In this and other studies (27, 28), the combination of PI3K inhibition by LY294002 with radiation has been evaluated in terms of decreased clonogenic capacity, but the underlying mechanisms of synergy remain unexplained. Microarray analysis provides an opportunity to survey more broadly the potential interactions of radiation and PI3K inhibition by assessing a large panel of gene expression levels. Our data shows that the addition of LY294002 to radiation treatment in HeLa cells does lead to altered expression of genes otherwise up-regulated or down-regulated by radiation alone.

Microarray analysis revealed four genes up-regulated by radiation that were not up-regulated when LY294002 was used in combination with radiation. Interestingly, ELK4 (an ETS domain protein), androgen receptor associated protein 24 (androgen receptor), muscarinic acetylcholine receptor M3, and HNRNP A2/B1 (an RNA binding protein) have been associated with prostatic, breast, and lung carcinogenesis and cell proliferation, respectively (35–38). This suggests that radiation-incited cellular responses that may be involved in promoting cell survival are being suppressed by the treatment with LY294002. Such suppression could contribute to the significant radiosensitization seen with the addition of this PI3K inhibitor.

Analysis of our data also shows that some genes significantly up-regulated by both LY294002 and radiation alone were not down-regulated when cells were treated in combination.
This finding may be explained by overlapping and redundant cellular processes affected by LY294002 and radiation. Alternatively, it may be due to the inherent weaknesses of our (in fact all) microarray methods and analyses. Failure to reveal a decrease in the combination treatment when one actually exists may be due to errors in the array spotting, sample hybridization, digital image analysis, as well as our statistical methodology, which excluded from analysis genes for which quality spots were not reproducible in all three triplicates. These findings are therefore currently being confirmed with quantitative PCR analysis of gene expression and by quantitation of protein levels.

In conclusion, we report the significant radiosensitization by LY294002 in cervical cancer cells exposed to radiation. This sensitization showed a significant time-dependent effect and was accompanied by significant decreases in cell growth and increases in apoptosis. Ongoing experiments in our lab are investigating the role of DNA damage signaling and repair machinery in mediating the radiosensitization of cervical cancer cells by the PI3K inhibitor LY294002. Once made adequately selective, molecularly targeted agents hold great promise in improving the radiosensitivity of solid tumors, such as cervical carcinomas, and therefore improving both local control and survival from this disease.

References