

Radiosensitization of cervical cancer cells via double-strand DNA break repair inhibition

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Abstract

Purpose. LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, has been found to radiosensitize various human cancer cells. However, its potential to act as an effective therapeutic agent is diminished by its toxicity levels. The purposes of this study were to determine the mechanism by which LY294002 radiosensitizes.

Materials and methods. Cell growth curves and clonogenic assays were performed with increasing LY294002 exposure times proximate to the radiation dose. Protein levels of downstream PI3K effectors were analyzed. Detection of phosphorylated histone H2AX (γ H2AX) was used to identify DNA double-strand breaks at various time points post-radiation.

Results. LY294002 significantly radiosensitized HeLa cervical cancer cells when administered for just 12 h following radiation. Cell growth curves also decreased with brief LY294002 application. DNA double-strand breaks are typically repaired within 2–6 h following radiation. Interestingly, at 48, 72, and 96 h post-irradiation, γ H2AX was still significantly elevated in cells radiated in combination with LY294002. Protein expressions of ATM and ATR downstream effectors showed no differences among the treated groups, however, DNA-PK activity was significantly inhibited by LY294002.

Conclusions. These results lead us to conclude that the central mechanism by which LY294002 radiosensitizes is via DNA-PK inhibition which induces DNA double-strand break repair inhibition. We are currently investigating radiosensitization induced by DNA-PK-specific inhibition in efforts to find a less toxic, yet equally effective, chemotherapeutic agent than LY294002.

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Introduction

Cervical carcinoma significantly affects women worldwide, especially in developing countries. It currently ranks as the second leading cause of cancer mortality in women (following

breast cancer). Approximately 500,000 cases of cervical cancer are diagnosed per year, with nearly 40% of those resulting in death [1]. Although the primary causative factor of cervical cancer is infection with human papilloma virus (HPV), specific aberrations of genes involved in control of cellular growth processes have also been implicated. It has been determined that by targeting these specific molecules, radiosensitivity and anti-proliferating responses can either be enhanced or diminished in cervical cancer cells [2].

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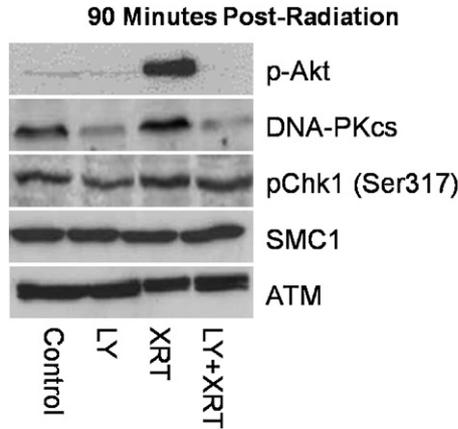


Fig. 1. DNA-PK and Akt Inhibition by LY. Protein extracts of cells treated with 25 μ M LY, 2 Gy radiation (IR), and in combination (LY+IR) at 90 min following radiation. Polyclonal anti-pan Akt was used as a loading control.

Previous studies have shown the phosphatidylinositol 3-kinase (PI3K) enzymes play key roles in the regulation of cell proliferation, differentiation, apoptosis, and cell cycle control [3–9]. Because of its important regulatory function as well as oncogenic properties, PI3K has been the source of much investigation [10–12]. LY294002 (LY), a PI3K inhibitor, has been shown to induce apoptosis, promote radiosensitivity, and arrest cell growth in cancer cells both *in vivo* and *in vitro* [6,13–15].

In this study, we examine the response of PI3K inhibition by LY in human cervical cancer cells exposed to ionizing radiation. Specifically, we sought to explore the mechanism by which LY radiosensitizes in order to find a more specific and less toxic therapeutic agent.

Materials and methods

Materials

LY294002 (a PI3K inhibitor) was obtained from Sigma Chemical Co. (St. Louis, MO). PhosphoPlus Akt[®] (Ser473) Antibody Kit and Phospho-Chk1/2 Antibody Sample Kit was obtained from Cell Signaling Technology, Inc. (Beverly, MA). SMC1 was obtained from Bethyl Laboratories, Inc. (Montgomery, TX). DNA-PKcs Antibody was obtained from BioLegend (San Diego, CA). The human cervical cancer cell line, HeLa, was kindly provided from Dr. Ray

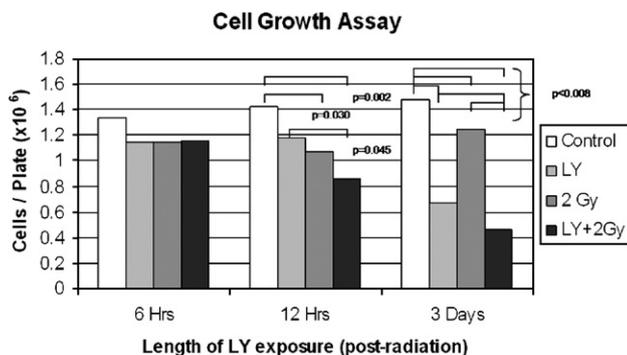


Fig. 2. Cell growth assay. Cells were treated with 25 μ M LY, 2 Gy radiation (IR), and in combination (LY+IR). Cell numbers were counted at 72 h following radiation. LY was removed at 6 h, 12 h, and 3 days following radiation.

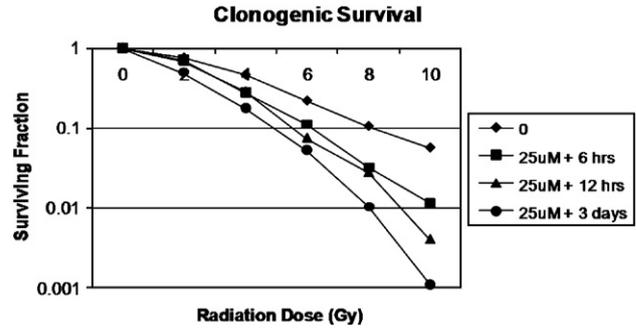


Fig. 3. Clonogenic survival. HeLa cells treated with 0–10 Gy radiation and 25 μ M LY. LY was removed at the varied times indicated following the radiation dose.

Lee, Huntsman Cancer Institute (Salt Lake City, UT). The Propidium Iodide (PI) stain was obtained from Molecular Probes, Inc. (Eugene, OR). The Anti-phospho-Histone H2A.X (Ser139), clone JBW301 was obtained from Upstate Biotechnologies (Charlottesville, VA). All cell culture reagents were obtained from Invitrogen Co. (Carlsbad, CA).

Cell culture

The HeLa cell line was maintained at 37 °C in DMEM supplemented with 10% fetal bovine serum in humidified 5% CO₂, 95% air. Culture medium was changed every 2–3 days. When cells reached confluence, they were disassociated by 0.05% trypsin–0.02% EDTA and replated. LY was diluted to 25 μ M concentration in media prior to each experiment from a stock solution of 10 mM in DMSO. The concentration of DMSO in both control and test groups did not exceed 0.5%. At the time of radiation treatment, cells were irradiated in their respective culture plates with a MARK 1 Cesium-137 Gamma Tissue Irradiator at a dose rate of 4.35 Gy/min.

Cell proliferation studies

For cell growth analysis, HeLa cells were plated at an initial density of 1.5×10^5 cells per 100-mm dish. LY was added 24 h after seeding. Control dishes received the same volume of the solvent DMSO. Irradiation (IR) was performed 30 min following addition of LY. Cells were harvested with trypsin–EDTA and cell number was determined using a Beckman Coulter Counter.

Flow cytometry for γ H2AX

Fixed cells were rinsed twice and rehydrated for 10 min in incubation buffer (0.5% BSA in 1 \times PBS) and were then centrifuged and resuspended in 200 μ l of mouse monoclonal anti-phospho-histone H2A.X antibody (1:500 dilution). Cells were incubated for 30 min at room temperature, rinsed twice, and resuspended in 200 μ l of secondary antibody, Alexa 488 goat anti-mouse IgG (H+L)(ab')₂ fragment conjugate (Molecular Probes; 1:200 dilution) for 30 min at room temperature. Cells were rinsed and resuspended in PBS before analysis on flow cytometer. DNA double-strand breaks were ascertained by the presence γ H2AX foci as determined by flow cytometry analysis.

Table 1

Comparison of α (alpha) and β (beta) components of survival curves with varying time administration of 25 μ M LY294002 (LY) combined with increasing doses of radiation (IR)

	α , Gy ⁻¹ (95% confidence interval)	β , Gy ⁻¹ (95% confidence interval)
IR alone (no LY)	0.097 (0.061–0.132)	0.024 (0.016–0.031)
6 h LY+IR	0.028 (–0.004–0.152)	0.055 (0.032–0.078)
12 LY+IR	0.069 (0.035–0.103)	0.061 (0.05–0.071)
72 h LY+IR	0.28 (0.27–0.29)	0.037 (0.033–0.041)

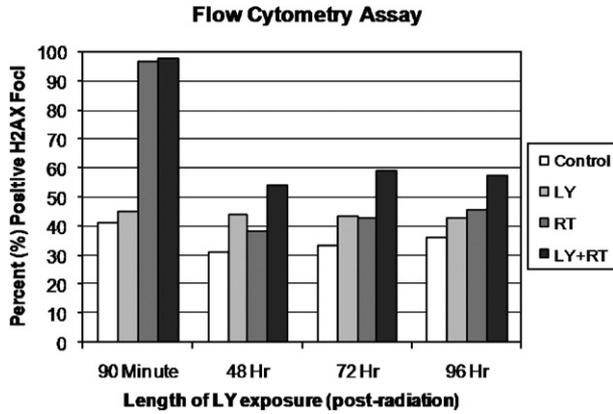


Fig. 4. Flow cytometry of γ H2AX showing prolonged expression. Cells were treated with 25 μ M LY, 2 Gy radiation (IR), and in combination (LY+IR).

Immunofluorescence microscopy

Cells were grown on coverslips and were fixed in 2% paraformaldehyde in PBS for 7 min and 50 mM NH₄Cl for 5 min. Cells were then permeabilized with 0.2% Triton X-100 for 3 min, rinsed twice with PBS, and then incubated for 1 h with anti- γ H2AX diluted 1:500 in 0.1% BSA in PBS. Cells were rinsed and incubated with Alexa 488 secondary antibody (1:200 dilution) with 0.5 μ g DAPI/ml in 0.1% BSA in PBS for 1 h at room temperature. Cells were rinsed and mounted on slides using FluorSave (CalBiochem) as the antifade mounting reagent, and sealed. Slides were then viewed using an Olympus Fluoview™ confocal microscope. Images were taken using Olympus Magnafire™ software.

Western blotting

Detection of phospho-Akt (Ser473) and phospho-Chk1 (Ser317), SMC1, and DNA-PKcs were analyzed by Western immunoblotting. Cells were treated with LY or DMSO 30 min prior to 2 Gy IR. LY exposure remained constant until 90 min post-radiation at which time protein was extracted. The cells were lysed in a SDS sample buffer and sonicated briefly. Samples were boiled, sheared, 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 the appropriate primary antibody and then reprobbed with a HRP-conjugated secondary antibody. Polyclonal anti-pan Akt was used 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. In order 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 performed by adding 25 μ M LY 30 min prior to 2 Gy IR as described before. At various time points following radiation, the media containing the LY was removed and fresh media was added. Cells were grown for 9–12 days with media changes every 3 days. Cells were then fixed with methanol and stained with methylene blue. Colonies containing more than 50 cells were evaluated by light microscopy and scored as survivors. In each case, the surviving fraction (SF) was calculated by dividing the number of colonies counted by the number of cells plated times the plating efficiency. Triplicate experiments were performed for each cell line.

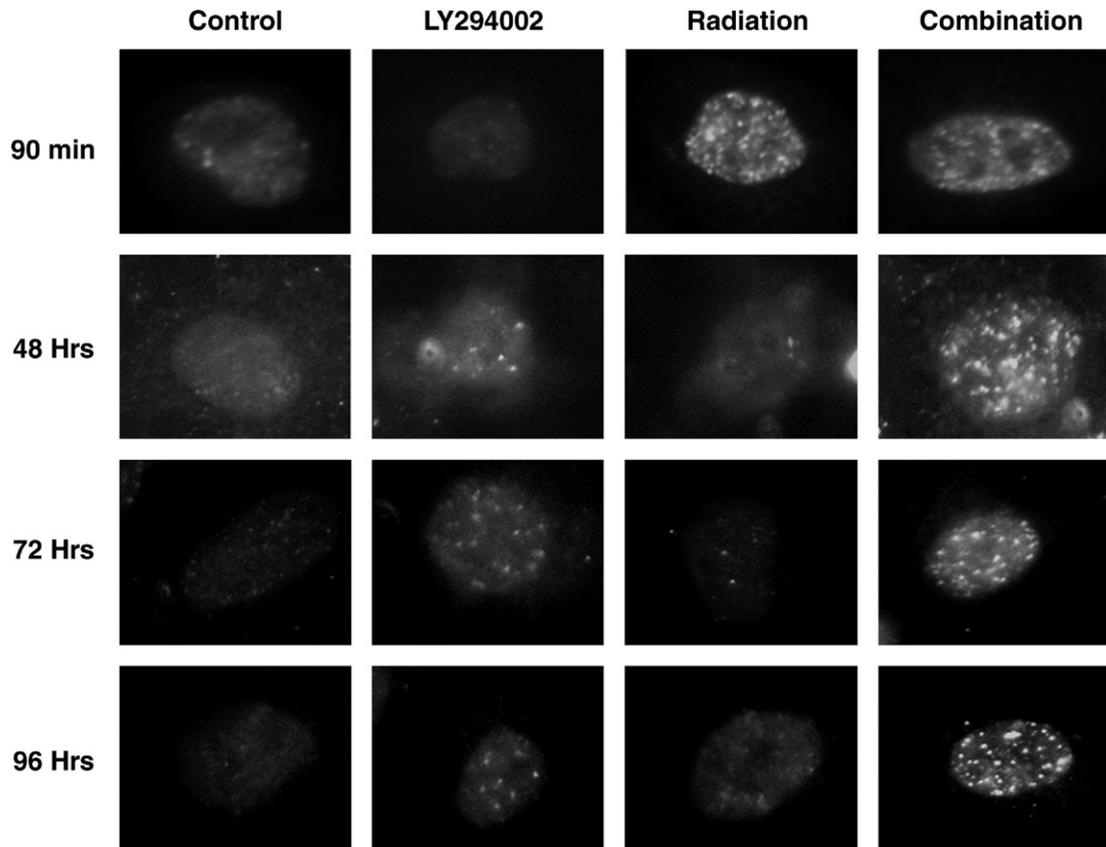


Fig. 5. Immunofluorescent microscopy of γ H2AX. Cells treated with 25 μ M LY and 2 Gy radiation. Prolonged expression is seen in all combination-treated cells at all times analyzed.

Table 2
Average γ H2AX foci per nucleus at various time points post-radiation

Time post-IR	Control	LY	IR	LY+IR
48 h	12.2	24.2	10.0	28.6
72 h	5.7	23.7	10.6	31.3
96 h	6.6	12.7	8.7	25.3

Irradiation (IR); LY294002 (LY).

Statistical analyses

The clonogenic survival data were fitted using the linear quadratic model $SF = \exp[-(\alpha)D - (\beta)D^2]$, where alpha equals the initial slope and beta equals the 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 (SAS) 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 performed 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, cell counts were evaluated between treatments by ANOVA at each time point with Dunnett's *t*-test to compare treatment differences with controls.

Results

DNA-PK and AKT inhibition by LY294002

Western Blot analysis shows significant increase of the Akt in radiation alone at 90 min (Fig. 1). However, when combined with LY, Akt activation is suppressed dramatically. The catalytic subunit of DNA-PK (DNA-dependent protein kinase) is also significantly inhibited with the addition of LY. Western blot analysis was also done looking at downstream effectors of ATM and ATR, SMC1 and p-Chk1 (Ser317), respectively, with no significant differences among the treatment groups at 90 min following radiation (Fig. 1).

Cell growth characteristics with LY removal

Fig. 2 illustrates the cell growth of HeLa cells collected 72 h post-radiation following LY exposure of 6, 12, and 72 h. With 6 h of drug exposure, no significant decrease was seen in cell growth. However, with only 12 h of treatment, the LY/IR-treated cells show significant decrease in growth when compared to control and LY-treated groups ($p=0.002$ and 0.045 , respectively), although no significant differences were seen among LY/IR and IR alone. For LY alone- and combination-treated cells, a significant decrease in cell growth was seen with 3 days of drug exposure ($p<0.008$ for all group comparisons).

Radiosensitization by LY measured by clonogenic assay

Clonogenic potential was assessed by colony formation assays following administration of LY and IR. Fig. 3 illustrates clonogenic assay results for 0 to 10 Gy IR with varied times of exposure to 25 μ M LY post-radiation. Our previous studies have shown that LY alone does not alter cell survival, however,

continuous treatment with 25 μ M LY has demonstrated significant radiosensitization of the HeLa cell line with a dose modification factor of 1.95 for 1 log cell kill [21]. Removal of LY at all time points tested post-radiation yielded significant radiosensitization across 0–10 Gy ($p<0.0001$). The dose modification factors for 1 log cell kill were 1.35, 1.40, and 1.61 for LY exposure of 6, 12, and 72 h post-treatment radiation. Of note, significant radiosensitization occurred despite limited exposure of LY for just 12 h post-radiation, indicating that LY-induced inhibition is mechanistically effective in the hours immediately following radiation. Analysis of α (alpha) and β (beta) slopes of the survival curves, as expressed by a linear quadratic equation, demonstrated alteration of both the α and β component even in the limited 6- and 12-h exposure groups (Table 1).

Prolonged expression of γ H2AX in cells treated with LY in combination with radiation

The combination of concurrent PI3K inhibition by 25 μ M LY and IR with a single fraction of 2 Gy significantly induced phosphorylation of histone H2AX from 90 min to 96 h post-radiation. This is shown graphically by flow cytometry analysis of the percentage of cells containing γ H2AX foci (Fig. 4) and visually by confocal microscopy (Fig. 5). As expected, IR-treated cells (with or without LY) showed significant increase in γ H2AX at 90 min. The number of double-strand breaks (or γ H2AX nuclear foci) was significantly increased at 48, 72, and 96 h in the combination-treated cells. Interestingly, the LY only-treated cells also demonstrated an increase in nuclear foci at 48 and 72 h but returned to control levels by 96 h (Table 2).

Cell growth inhibition in combined treated cell groups

Cell growth in all treated groups was significantly inhibited at all time points investigated (Fig. 6). However, the groups treated with LY and LY with IR had almost complete growth inhibition when compared to control and IR-treated cells possibly indicating either stalled cell cycle progression or DNA double-strand break repair inhibition.

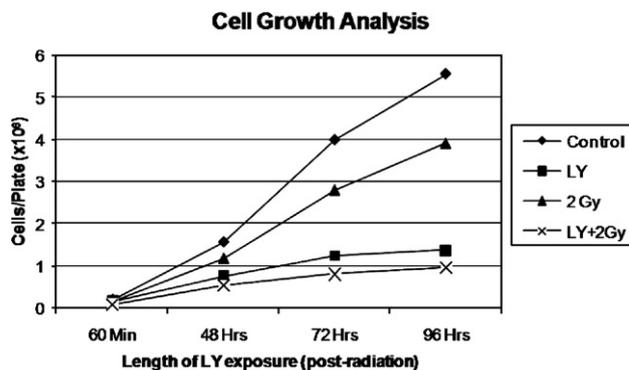


Fig. 6. Cell growth analysis. Cells treated with 25 μ M LY, 2 Gy radiation (IR), and in combination (LY+IR). LY treatment was administered for the duration of the time indicated. Growth was inhibited in all combination-treated cells at all times analyzed.

Discussion

PI3K inhibition of LY has shown potent radiosensitization in cervical cancer cell in our laboratory [16] and others' [13–15]. In this study, we show that LY administration is effective even when briefly administered during and directly after radiation. As a broad inhibitor across all major classes of PI3Ks and PI3K-like kinases (PIKKs), the mechanisms of LY radiosensitization have not been fully characterized. We also report in this study prolonged presence of radiation-induced DNA double-strand breaks implicating the inhibition of double-strand break repair as a major mechanism of LY radiosensitization. The families of PIKK enzymes (ATM, ATR, and DNA-PK) are all involved in detection of DNA damage, activation of DNA repair machinery and, in turn, the linking of this to cell cycle control [10,17–20]. In the current study, despite PI3K inhibition, cervical cancer cells were able to detect double-strand breaks as evidenced by formation of γ H2AX foci at 60 min post-radiation. It has been well documented that ATM is the major kinase responsible for modifying H2AX upon IR [21,22]. However, even in the presence of the LY inhibitor, early H2AX phosphorylation is able to be carried out. This may be possible via the ability of ATM and DNA-PK to act in a redundant, overlapping manner to phosphorylate H2AX [23]. To support this theory, we have shown that ATM is not significantly inhibited at these doses of LY in HeLa cervical cancer cells.

DNA-PK has been strongly implicated in double-strand break repair [24,25] and may have a regulatory role in matching correct double-strand break ends to be joined. The catalytic subunit of DNA-PK (DNA-PKcs) shares sequence homology in its kinase domain with PI3K and is inhibited by LY in an ATP-competitive manner [26]. DNA-PK inhibition via LY treatment on *Xenopus* egg extracts inhibits end-joining of cohesive 3'-hydroxyl ends of DNA and suppresses the processing of damaged DNA ends prior to joining [27]. Okayasu et al. suggest that LY radiosensitization may be due in part by DNA-PK inhibition resulting in DNA BSB repair inhibition as reflected in the excess number of interphase chromosome fragments in irradiated cells pretreated with LY [28].

Histone H2AX phosphorylation has long been used as a standard method for detecting double-strand breaks at the site of DNA damage. It is rapidly phosphorylated (within seconds) at serine 139 when double-strand breaks are introduced in mammalian cells resulting in discrete foci at the DNA damage sites [29,30]. These foci continue to grow for approximately 1 h following radiation and then dissipate over time, correlating with the rejoining of DNA breaks. Many studies have shown that the residual level of γ H2AX measured 24 h post-radiation and longer, as seen in this particular study, directly correlates with radiation sensitivity [31–33].

The significantly prolonged expression of γ H2AX in HeLa cells treated with LY in combination with radiation, along with radiosensitization induced with brief LY application immediately following radiation, as seen in these studies

may result from impaired DNA double-strand break repair and/or cell cycle arrest via DNA-PK inhibition. Ongoing work in our lab aims to differentiate these two mechanistic pathways. Delineation of these mechanisms and LY radiosensitization may allow for further targeting of relevant pathways. These findings support future investigation of mechanistic and therapeutic uses of DNA-PK-specific inhibitors in combination with radiation therapy for carcinoma of the cervix.

Acknowledgment

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