Silibinin Preferentially Radiosensitizes Prostate Cancer by Inhibiting DNA Repair Signaling

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Abstract

Radiotherapy, a frequent mode of cancer treatment, is often restricted by dose-related toxicity and development of therapeutic resistance. To develop a novel and selective radiosensitizer, we studied the radiosensitizing effects and associated mechanisms of silibinin in prostate cancer. The radiosensitizing effect of silibinin with ionizing radiation (IR) was assessed on radioresistant prostate cancer cell lines by clonogenic, cell cycle, cell death, and DNA repair assays. Tumor xenograft growth, immunohistochemical (IHC) analysis of tumor tissues, and toxicity-related parameters were measured in vivo. Silibinin (25 \textmu mol/L) enhanced IR (2.5–10 Gy)-caused inhibition (up to 96%, \(P < 0.001\)) of colony formation selectively in prostate cancer cells, and prolonged and enhanced IR-caused G2–M arrest, apoptosis, and ROS production. Mechanistically, silibinin inhibited IR-induced DNA repair (ATM and Chk1/2) and EGFR signaling and attenuated the levels of antiapoptotic proteins. Specifically, silibinin suppressed IR-induced nuclear translocation of EGFR and DNA-PK, an important mediator of DSB repair, leading to an increased number of γ-H2AX (ser139) foci suggesting lesser DNA repair. In vivo, silibinin strongly radiosensitized DU145 tumor xenograft inhibition (84%, \(P < 0.01\)) with higher apoptotic response (10-fold, \(P < 0.01\)) and reduced repair of DNA damage, and rescued the mice from IR-induced toxicity and hematopoietic injury. Overall, silibinin enhanced the radiotherapeutic response via suppressing IR-induced prosurvival signaling and DSB repair by inhibiting nuclear translocation of EGFR and DNA-PK. Because silibinin is already in phase II clinical trial for prostate cancer patients, the present finding has translational relevance for radioresistant prostate cancer. Mol Cancer Ther; 14(12): 1–13. ©2015 AACR.

Introduction

Radiotherapy is a frontline treatment option in prostate cancer, next only to radical prostatectomy, with 25\% of men ages 18 to 65 years and 42\% of men ages 65 to 74 years undergoing radiotherapy (1). However, the positive facets of radiotherapy are moderated by its detrimental consequences on normal dividing cells and emergence of therapeutic resistance (2). Hence, development of novel radiosensitizing agents with a capacity to improve therapeutic index mandates urgent attention.

The major mechanisms responsible for radiotherapeutic resistance are the activation of prosurvival and DNA repair pathways in response to IR (3, 4). In general, IR-induced cell death is mediated by induction of double-stranded breaks (DSB) in DNA, which leads to faulty cell division and death by mitotic catastrophe. However, in response to IR, the damaged cells also activate their DNA repair machinery, including ATM and DNA-PK, which reduces the extent of radiation-induced damage and resultant death (5). IR also activates mitogenic signaling especially EGFR (6), and IR-induced EGFR nuclear translocation, which activates DNA repair pathways (7, 8). Understandably, these mechanisms are unfavorable to the use of radiation in cancer treatment, and suggest that agents that could inhibit EGFR-mediated signaling and/or DNA repair following radiotherapy could be successful radiosensitizers.

Here, we have evaluated the radiosensitizing properties of a plant flavonoid silibinin in prostate cancer, a malignancy with late-stage radioresistance. Silibinin possesses strong anticancer activity against prostate cancer in preclinical studies and is currently in phase II clinical trial against prostate cancer (9, 10). Subsequently, we rationalized that silibinin could enhance radioresponse and may have additional translational potential. Indeed, our results suggested that silibinin preferentially sensitizes prostate cancer cells to IR, reduces IR-induced systemic injury and tissue toxicity and in mice, and enhances radiotherapeutic index for prostate cancer.

Materials and Methods

Cell lines and reagents

Human prostate carcinoma DU145, PC-3, and 22RV1 cells, mouse keratinocyte JB6 cells, human lung cancer A549 cells were from the ATCC. DU145, 22RV1, and A549 cell lines were characterized by short tandem repeat analysis in August 2013, and PC-3 in July 2015. JB6 cells were procured in January 2010. HEK-293 cells were purchased from National Centre for Cell Science in August 2010. Initially, cells were grown and frozen in liquid...
nitrogen. Cells grown from a vial were always monitored for their morphology and used for the experiments within 5 months. Other cell culture materials were from Himedia. Silibinin (MW = 482.4), RPMI media, propidium iodide (PI), DCFH-DA, antibodies to Cdc25C, DNA-PK, β-actin were obtained from Sigma-Aldrich Chemical Co. Antibodies for Cyclin B1, Cdc-2, PCNA and survivin, phospho-EGFR, ATM, phospho-Chk1, phospho-Chk2, Chk1, Chk2 and anti-rabbit peroxidase–conjugated secondary antibody were from Cell Signaling Technology. Anti-tubulin and anti-mouse peroxidase antibody were from Cell Signaling Technology. Anti-tubulin and anti-mouse peroxidase–conjugated secondary, goat anti-rabbit IgG were from Santa Cruz Biotechnology. ECL detection system was from Millipore.

Cell culture and treatments

DU145, PC-3, A549, and 22RV1 cells were cultured in RPMI-1640 medium with 10% FBS and penicillin–streptomycin at 37°C in 5% CO2 incubator. HEK-293 cells were grown in DMEM with 10% FBS and IB6 cells in MEM medium with 5% FBS. Cells were treated with varying doses of radiation (2.5–10 Gy) and/or silibinin (25–100 μmol/L), which was dissolved in DMSO. The treatment time varied from 3 to 72 hours depending upon the experiment. An equal volume of DMSO (0.1% v/v) was present in each treatment.

Irradiation protocol

The cells were irradiated in 60Co gamma chamber (Model 4000A, Bhabha Atomic Research Centre) at a dose rate of 4.6 Gy/min. For the animal experiment, mice were irradiated using RS 2000 Biological Irradiator (Rad Source Technologies) housed at Anschutz Medical campus. The dose rate of the irradiator was 1.34 Gy/min. The silibinin and IR treatments were started when the tumor volume reached 200 mm3. Silibinin was given as 200 mg/kg body weight per day in 0.5% CMC by oral gavage.

Colony formation assay

Cells were seeded in 6-well plates (500 cells/plate). After 24 hours of plating, cells were treated with respective doses of radiation and/or silibinin (co-treatment) and eventually cultured for 10 days; the colonies formed were fixed with ice-cold methanol:glacial acetic acid (3:1) for 10 minutes; and stained with 1% crystal violet. Plating efficiency was calculated by dividing the average number of colonies per well by the amount of cells plated. Survival fractions were calculated by normalization to the plating efficiency of appropriate control groups. Dose-enhancement ratio (DER) was calculated as the ratio of radiation dose without silibinin to dose of radiation with silibinin required to achieve the same amount of cell kill. If DER is >1, the agent is considered to be radiosensitizing, whereas a value <1, the agent is considered to be radioprotective.

Cell-cycle analysis by flow cytometry

Cell-cycle distribution was analyzed by flow cytometry using BD FACS Calibur by BD Biosciences and data were analyzed with ModFit LT software, as described in earlier studies (11).

Reverse transcriptase PCR

Cells were seeded and grown in 100-mm culture plates to 70% confluency under regular growth conditions and were treated with silibinin (25 μmol/L) and/or radiation (3 Gy) in 10% serum supplemented RPMI-1640 medium. Total RNA was isolated using TRizol reagent and cDNA was synthesized as described earlier. This was followed by standard PCR reactions using gene-specific forward and reverse primers (Supplementary Methods). PCR products were analyzed by running on 1% agarose gel stained with ethidium bromide and photographed under low intensity UV in GelDoc system (Applied Biosystems).

BrdUrd incorporation assay

Briefly, the cells were cultured in 96-well plates at a density of 5,000 cells/100 μL/well in complete growth media. After 48 hours of respective treatments, the cells were labeled with bromodeoxyuridine (BrdUrd) using the Cell Proliferation ELISA, BrdUrd (colorimetric) Kit (Roche Applied Science), and the percent BrdUrd incorporation was measured in each treatment, as per the manufacturer’s protocol.

Analysis of ROS levels

Cells were treated with DMSO or 25 μmol/L silibinin and/or 5 Gy for 12, 24, 48, and 72 hours. Cells were incubated with 20 μmol/L DCF-DA during the last 30 minutes of treatment at 37°C. The probe was washed off with PBS and the cells were trypsinized, resuspended in PBS, and analyzed for DCF-positive cells by flow cytometry.

DNA fragmentation assay

At the end of the treatment time, cells were gently scraped and centrifuged at 1,000 × g for 5 minutes at 4°C following which the cells were lysed by adding 500 μL of the lysis buffer containing 1% NP-40, 20 mMmOL/L EDTA, 5 mMmOL/L Tris-HCl-pH, 8.0. The lysed sample was then centrifuge at 12,000 × g at 4°C for 20 minutes and the supernatant was collected and treated with RNase at 37°C for 1 hour. The fragmented DNA was then extracted by the phenolchloroform method. Fragmented DNA was precipitated overnight at −20°C after adding 30 μL of 5 mol/L NaCl to a final concentration of 300 mMmOL/L and 2.5 volume of ice-cold 100% ethanol. The pellet obtained after centrifugation, was washed with 70% ethanol, air dried and resuspended in TE buffer, and run on a 1.2% agarose gel containing 0.5 μg/mL ethidium bromide.

AO-EtBr apoptosis assay

After the completion of desired treatments, total cells were collected by centrifugation and added with 50 μL of staining solution containing the acridine orange (AO) and EtBr mix (100 μg/mL each) in PBS. Then cells were put on a slide and visualized under 100 × field of a fluorescent microscope. A minimum of 250 cells were scored and the percentage of apoptotic cells were determined. Early apoptotic cells showed green nuclei with condensed chromatin whereas late apoptotic cells showed orange nuclei with condensed chromatin.

Immunoblot analysis

Prostate cancer cells were grown in regular serum conditions to 70% confluency and treated with the desired doses of silibinin and/or radiation. At the end of the treatment time periods, whole-cell lysates were prepared in nondenaturing lysis buffer as published recently (12). Cytoplasmic and nuclear
followed by 3,3'-diaminobenzidine (DAB) staining, as previously described. Biotinylated secondary antibodies used were horseradish peroxidase conjugated. The Student t test was used for statistical significance (P < 0.05). The paired Student t test was used for tumor volumes.

Results

Silibinin preferentially radiosensitizes prostate cancer cells

Effect of silibinin in sensitizing prostate cancer cells to IR was assessed using two radioresistant human prostate cancer cell lines DU145 and PC-3, using clonogenic survival assays. PC-3 were more sensitive to IR treatment than DU145, showing 47% decrease in colony formation compared with 32% in DU145 at 5 Gy dose (Fig. 1A and B). IR (2.5–10 Gy) inhibited colony formation by 17% to 68%, which increased to 29% to 92% (P < 0.001) in combination with 25 μmol/L silibinin in DU145 cells (Fig. 1A). Similar results were observed for PC-3 cells (Fig. 1C). The DER at 50% inhibition in colony formation was 1.67 for DU145 and 1.4 for PC-3 cells. Silibinin also radiosensitized other type of cancer cells, for example, human lung carcinoma A549 cells, with a DER of 1.6 (Fig. 1D). More importantly, in non-neoplastic human embryonic kidney cells (HEK-293), similar treatment with IR and/or silibinin did not radiosensitize the cells; in fact, it resulted in radioprotection with a DER of 0.83 at 50% inhibition (Fig. 1E). In radioresponsive 22RV1 cells, combination treatment did not show any significant increase in radiosensitivity (Supplementary Fig. S1).

Silibinin enhanced and prolonged IR-induced G2-M arrest of prostate cancer cells

IR alone increased G2–M cell population from 20% in control to 37%, which was further increased to 59% (P < 0.001) when combined with 25 μmol/L silibinin at 24 hours, and in the combination treatment, the effect was prolonged (P < 0.01) even until 48 hours (Fig. 2A, left and Supplementary Fig. S2). A similar trend in cell-cycle effects was observed in PC-3 cells with combination treatment (Fig. 2A, right). Concurrently, the expression levels of Cyclin B1 and Cdc2 decreased as early as 6 hours following the combination treatment (Fig. 2B). This effect was sustained until 48 hours, where we also observed IR-induced increase in Cdc25C, which was strongly decreased in the IR plus silibinin treatment, supporting prolonged G2–M block in the combination treatment (Fig. 2B, right and Supplementary Fig. S3A). In HEK-293 cells, combining SB with radiation did not show significant difference in gene expression of cell-cycle regulators as compared with radiation treatment alone, which again suggests that silibinin shows a differential response (Supplementary Fig. S3A).

Silibinin strongly inhibited cancer cell proliferation following IR exposure and downregulated IR-induced expression of prosurvival molecules

Following irradiation, a fraction of cells that are lethally damaged undergo apoptosis, but the remaining cells that are sublethally irradiated try to evade apoptosis, by activating a prosurvival response. After 48 hours of treatment, BrdUrd incorporation and statistical analyses

Bands on X-ray films were scanned and their mean density was analyzed by ImageJ (NIH, Bethesda, MD). Densitometry data, which is represented below the bands, are the “fold change” as compared with respective DMSO control, after normalization with respective loading controls (β-actin). The data were statistically analyzed using the Jandel Scientific Sigma Stat 3.5 software.

Immunohistochemical analysis of tumors

After 24 hours following the final irradiation and silibinin treatment, the mice were euthanized and tumors were dissected out, weighed, fixed in formalin, and further processed and embedded in paraffin. Paraffin-embedded tissue sections were deparaffinized and stained using specific primary antibody followed by 3,3′-diaminobenzidine (DAB) staining, as previously described. Biotinylated secondary antibodies used were rabbit anti-mouse IgG (1:200; Dako) and goat anti-rabbit IgG (1:200; Santa Cruz Biotechnology). Apoptotic cells were identified by TUNEL staining using Dead End Colorimetric TUNEL System (Promega Corp.) as published (13). The percentage of Ki-67, TUNEL, pChk2, pH2AX-positive cells were quantified by counting brown-stained cells within total number of cells at five arbitrarily selected fields from each tumor at 400× magnification.

Densitometric and statistical analyses

Bands on X-ray films were scanned and their mean density was analyzed by ImageJ (NIH, Bethesda, MD). Densitometry data, which is represented below the bands, are the “fold change” as compared with respective DMSO control, after normalization with respective loading controls (β-actin). The data were statistically analyzed using the Jandel Scientific Sigma Stat 3.5 software.

In vivo tumor xenograft study

Athymic (nu/nu) male nude mice (NCI, Frederick, MD) approved by the Institutional Animal Care and Use Committee of the University of Colorado Denver were s.c. injected in the right flank with 6 × 10^6 DU145 cells mixed with Matrigel. From the day following xenograft implantation, mice were monitored regularly for tumor growth and once the tumors reached approximately around 200 mm^3, the mice were randomly divided into four groups and respective treatments were given. For the radiation alone group, IR treatment (2.5 Gy/dose) was given at an interval of 2 days, until a cumulative dose of 15 Gy was achieved (day 15). Mice were anesthetized with ketamine/xylazine before radiation and positioned under a lead shield such that only the tumor-bearing flank was exposed. Silibinin (200 mg/kg) was given in 0.5% CMC (w/v) to animals as oral gavage just before the (200 mg/kg) was given in 0.5% CMC (w/v) to animals as oral gavage just before the [10% (v/v) FBS, 0.3% (w/v) TritonX-100] overnight at 4°C, and then incubated with the primary rabbit anti-pEGFR (1:200) or DNA-PKcs (1:250) overnight at 4°C, and then incubated with the secondary antibody anti-rabbit IgG (1:500) for 1 hour, and finally counterstained with 300 nmol/L DAPI as published earlier (12). Cells were examined using a confocal microscope at our Central Instrument Facility in the school.

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Immunofluorescence staining assay

Cells were grown on coverslips in 24-well plate, washed with PBS, fixed in 4% formaldehyde for 15 minutes, and permeabilized with 0.3% TritonX-100 for 15 minutes. Cells were blocked with [10% (v/v) FBS, 0.3% (w/v) TritonX-100] overnight at 4°C, and then incubated with the primary antibody anti-pEGFR (1:200) or DNA-PKcs (1:250) overnight at 4°C, and then incubated with the secondary antibody anti-rabbit IgG (1:500) for 1 hour, and finally counterstained with 300 nmol/L DAPI as published earlier (12). Cells were examined using a confocal microscope at our Central Instrument Facility in the school.

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Immunohistochemical analysis of tumors

After 24 hours following the final irradiation and silibinin treatment, the mice were euthanized and tumors were dissected out, weighed, fixed in formalin, and further processed and embedded in paraffin. Paraffin-embedded tissue sections were deparaffinized and stained using specific primary antibody followed by 3,3′-diaminobenzidine (DAB) staining, as previously described. Biotinylated secondary antibodies used were rabbit anti-mouse IgG (1:200; Dako) and goat anti-rabbit IgG (1:200; Santa Cruz Biotechnology). Apoptotic cells were identified by TUNEL staining using Dead End Colorimetric TUNEL System (Promega Corp.) as published (13). The percentage of Ki-67, TUNEL, pChk2, pH2AX-positive cells were quantified by counting brown-stained cells within total number of cells at five arbitrarily selected fields from each tumor at 400× magnification.

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decreased by 36% (P < 0.001) in silibinin with IR as compared with IR alone (16%) in DU145 cells, whereas in PC-3 cells, combination treatment resulted in 39% (P < 0.01) inhibition versus 30% in IR alone (Fig. 2C). This inhibition was aided via decreased expression of both PCNA and survivin, which did not change with IR alone in DU145 cells (Fig. 2D). In PC-3 cells, IR appeared to posttranscriptionally modify and increase protein levels of PCNA and survivin, which were decreased by the combination treatment (Fig. 2D and Supplementary Fig. S3B).

Silibinin enhanced IR-induced ROS production and led to prolonged oxidative stress

Although, silibinin has antioxidant activity, it is now well documented that many polyphenols, including silibinin, also behave as pro-oxidants under certain conditions (14, 15). We found that IR showed an established distribution pattern of ROS production during 12 to 72 hours of treatments with a peak at 24 hours, whereas silibinin showed peak of ROS production at approximately 48 hours, with 27% positive cells (Fig. 2E, left). In the combination treatment, there was a dramatic increase in the ROS-positive cells with a peak at 24 hours (61%, P < 0.001; Fig. 2E; Supplementary Fig. S4A and S4B). Combination of silibinin with IR also led to reduction in the mRNA expression of antioxidant enzymes including SOD1, SOD2, Catalase, and GST, supporting the data showing enhanced oxidative stress in these cells (Supplementary Fig. S4C).

Because instead of radiosensitization, we had observed radioprotection of HEK-293 cells by silibinin, we examined whether this differential effect could be facilitated via the modulation of redox status. Surprisingly, presence of silibinin with IR showed an inverse effect on HEK-293 cells to that of cancer cells. Silibinin treatment alone showed 7% ROS-positive
Figure 2.
Silibinin potentiates IR-induced G2–M arrest and inhibition in cell proliferation, and augments oxidative stress selectively in prostate cancer cells. DU145 and PC-3 cells were exposed to IR with or without silibinin (SB). After treatment time points, cells were processed for cell-cycle analysis using saponin-PI staining. A, quantitative data showing cell-cycle distribution in DU145 (left) and PC-3 cells (right) after treatment with IR (5 Gy) and/or SB (25 μmol/L). B, Western blots for G2–M cell-cycle-related proteins at 3, 6, and 48 h. Cell proliferation rate in cells was assessed by BrdUrd incorporation assay. C, the percentage of BrdUrd incorporation was calculated with respect to control after 48 hours treatment in both DU145 and PC-3 cells. D, RT-PCR and immunoblotting analysis of PCNA and survivin proteins after 48 hours treatment. E, for oxidative stress analysis, cells were analyzed for DCF fluorescence by flow cytometry, after treatment with IR (5 Gy) and/or SB (25 μmol/L). The percentage of positive cells was those with a fluorescent intensity >10^4 on the histogram. Graph showing change in the DCF-positive cells in different groups after 12 to 72 hours of treatment of DU145 cells. Bar diagram showing DCF-positive cells for DU145 and HEK-293 cells after 24 hours of treatment. P < 0.001 (*) and P < 0.01 (#) compared with respective control or indicated treatment.
Figure 3.
Silibinin potentiates radiation-induced apoptosis and attenuates DNA repair activation signaling in prostate cancer cells. A, representative pictures (left) and quantitative data (right) showing DNA laddering in DU145 and PC-3 cells treated with IR (5 Gy) and/or silibinin (25 μmol/L) for 48 hours. B, graphical data depicting the percentage of cells positive for apoptosis after AO–EtBr staining. C, Western blot analysis of Bcl-2 and cleaved caspase-3 in DU145 and PC-3 cells after 48 hours of treatment. D, immunoblotting for damage signaling molecules activated in response to IR (5 Gy) and/or SB (25 μmol/L). E, RT-PCR for cell-cycle check-point regulators Chk1 and Chk2; and F, phospho/total Chk1 (threonine 345 and serine 296) and Chk2 levels (threonine 68) in DU145 cells. *P < 0.001 (∗) and P < 0.05 ($) compared with respective control or indicated treatment.
Figure 4.
Silibinin inhibits nuclear translocation of EGFR and DNA-PK in cancer cells and reduces repair of pH2A.X (Ser 139) foci in prostate cancer cells. A, confocal microscopy showing distribution of EGFR (red) in DU145 cells in response to IR (5 Gy) and/or SB (25 μmol/L). B, immunoblotting for EGFR in cytoplasmic and nuclear fractions after 3 hours of treatments; tubulin and lamin were used as loading control for cytoplasmic and nuclear compartments, respectively. C, confocal microscopy showing distribution of DNA-PK (red), nucleus (Sytox-green) in DU145 cells in response to IR (5 Gy) and/or SB (25 μmol/L) at 3 hours; and D, representative images of pH2A.X foci (red) in the nucleus at 12 hours and quantitation of number of pH2A.X foci after 6 and 12 hours of treatments in DU145 cells. P < 0.001 (*) and P < 0.05 (#) compared with respective control or indicated treatment.
Figure 5.

Silibinin treatment enhances radiation-induced tumor growth inhibition of human prostate cancer DU145 xenograft in athymic nude mice. A, diagrammatic representation of the time line followed for the tumor study. Mice were s.c. injected with DU145 cells ($6 \times 10^6$) mixed with Matrigel (1:1) and monitored for tumor growth until the tumor size reached approximately 200 mm$^3$. Then mice were treated with IR (2.5 Gy) with a gap of 2 days between two IR fractions, with or without SB (200 mg/kg), which was given 5 days per week. Control and IR-alone group of mice were gavaged with 0.5% CMC in saline. The treatment was continued until the cumulative irradiation dose reached 15 Gy. Twenty-four hours after the final fraction of IR (day 16), the tumors were excised and processed further for immune-histochemical staining. B, tumor volume/mouse as a function of cumulative radiation dose; C, tumor weight/mouse at the end of study; D, mean body weight per mouse; and E, average diet consumption per mouse per day were analyzed as detailed in Materials and Methods. Data, mean ± SE from 8 mice in each group. Effect of IR and/or silibinin was also checked on the hematopoietic system at the end of the experiment, mean WBC count per mouse (F), mean lymphocyte count/mouse (G), and represents mean platelet count/mouse (H); $P < 0.01$ ($\#$) and $P < 0.001$ (*) compared with respective control.
Silibinin Preferentially Radiosensitizes Prostate Cancer

A

Ki-67

pChk-2(T68)

pH2A.X(S139)

TUNEL

Control                     SB                             IR                         IR+SB

B

% Ki-67-positive cells

C

% P-Chk2(T68)-positive cells

D

% pH2A.X(S139)-positive cells

E

% TUNEL staining apoptotic cells

F

Activated by IR in PCa cells

Mitogenic and prosurvival signaling

Enhanced cell growth and proliferation

Inhibition of apoptosis

G2-M cell-cycle arrest (ATM-Chk1/2)

DNA repair and radiosensitivity

Radiosensitivity in cancer cells

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Silibinin Preferentially Radiosensitizes Prostate Cancer

A

Control                     SB                             IR                         IR+SB

Ki-67

pChk-2(T68)

pH2A.X(S139)

TUNEL
cells, whereas in IR alone, there were 25% ROS-positive cells at 48 hours (Fig. 2E, right and Supplementary Fig. S4D). However, combining silibinin with IR significantly reduced ROS-positive cells to 7.6% ($P < 0.001$).

**Silibinin enhanced IR-induced apoptosis**

Because prosurvival molecules were suppressed and ROS level was enhanced in combination treatment, we assessed whether it led an increase in radiation-induced apoptosis. Compared with either silibinin or IR, an intense DNA laddering (>2-fold) in combination treatment was observed in both DU145 and PC-3 cells (Fig. 3A). AO–EtBr assay also showed an increased in apoptosis from 25% to 27% in IR alone to 44% to 46% ($P < 0.001$) in combination after 48 hours in both prostate cancer cells (Fig. 3B). Bcl-2 overexpression, a major player in the development of radioresistant phenotype (16), was upregulated by IR in DU145 cells, whereas combining it with silibinin led to a profound decrease in Bcl-2 expression in DU145 cells with a moderate effect on PC-3 cells (Fig. 3C).

**Silibinin augmented the therapeutic efficacy of irradiation by inhibiting DNA repair**

One of the major mechanisms for acquired radioresistance in cancer cells is the DNA repair, DNA being the principle target of radiation-induced damage (17). Our results revealed that IR enhances ATM expression as early as 3 hours but in combination treatment, especially at 6 hours, it downregulated the expression of ATM in DU145 cells (Fig. 3D). Also, the phosphorylated level of EGFR (Y1068) was enhanced with IR, which was reduced by silibinin treatment. Chk1 and Chk2, the downstream effectors of ATM involved in activation of DNA repair (18), were also induced by IR showing an increase in mRNA levels (Fig. 3E) and enhanced phosphorylation of Chk1 (S345 and S269) and Chk2 (T68). These IR-induced levels were downregulated in the combination treatment (Fig. 3F).

**Silibinin inhibited IR-induced nuclear translocation of EGFR**

The role of nuclear EGFR in development of radioresistance by acting as a mediator for DNA repair is gaining grounds (6, 8). Furthermore, IR-induced EGFR activation is a prominent contributor to radioresistance. Because we observed inhibition of IR-activated EGFR, we further analyzed whether silibinin can alter IR-induced nuclear translocation of EGFR in prostate cancer cells. IR exposure of DU145 cells resulted in nuclear translocation of EGFR (red) at 3 hours that was almost completely inhibited by silibinin and EGFR localization was limited to the cytosol (Fig. 4A). This was further confirmed by measuring EGFR protein levels in cytosolic and nuclear fractions (Fig. 4B). We also checked the effect of these treatments on nuclear translocation of EGFR in non-neoplastic JB6 mouse keratinocyte cells (Supplementary Fig. S5). Compared with prostate cancer cells, we did not observe considerable reduction in the nuclear localization of EGFR in JB6 cells, suggesting that this effect of silibinin may be selective to neoplastic cells.

**Silibinin inhibited IR-induced nuclear translocation of DNA-PK and prolonged the presence of pH2A.X foci**

Confocal microscopy showed that after IR treatment, most of DNA-PK was localized into the nucleus. However, when cells were treated with silibinin and IR, like EGFR, DNA-PK too remained excluded out of the nucleus, thereby blocking it from carrying out its DNA repair function (Fig. 4C). To further support that silibinin inhibits IR-induced DNA repair signaling, pH2A.X foci were assessed as indicator of DNA damage. We observed that IR exposure increased pH2A.X foci at 6 hours, which was reduced by 12 hours (59% decrease, $P < 0.001$), whereas in presence of silibinin, it was increased by 38% (Fig. 4D). Furthermore, by 12 hours in the presence of silibinin, the number of pH2A.X foci was more than 2.5-fold ($P < 0.001$) from that of IR alone (Fig. 4D). This persistence of pH2A.X foci levels in combination as compared with IR alone suggests that silibinin-mediated radiosensitization involves an inhibition of repair of IR-induced DNA damage.

**Silibinin enhanced radiation-induced tumor growth inhibition and protected the normal tissue from radiation injury**

After establishing the radiosensitizing properties of silibinin in vitro, we substantiated these findings in the DU145 xenograft model. Once the tumors reached approximately 200 mm$^3$, mice were treated with IR and/or silibinin, as detailed in the Materials and Methods (Fig. 5A). Silibinin and IR inhibited tumor growth (volume) by 56% ($P < 0.01$) and 61% ($P < 0.01$) from control, respectively; however, their combination led to 84% ($P < 0.001$) growth inhibition when compared with control (Fig. 5B). Similarly, tumor weight was decreased by 46% ($P < 0.01$), 43% ($P < 0.01$), and 82% ($P < 0.001$) in silibinin, IR and IR with silibinin-treated groups from control, respectively (Fig. 5C). The tumor volume and weight were decreased by 61% ($P < 0.01$) and 69% ($P < 0.01$) in combination when compared with IR alone, respectively.

IR treatment alone led 13% and 30% decrease in body weight and diet consumption, respectively, at the end of treatment; however, silibinin treatment reversed these losses by 8% and 19% ($P < 0.01$ for both), respectively (Fig. 5D and E). IR leverages heavy toxicity to the hematopoietic system (18). We observed that total WBC, neutrophil, monocyte, and platelet counts were reduced by 33% to 50% by IR; however, treatment with silibinin completely blocked ($P < 0.01–0.001$) these adverse effects of IR on hematopoietic system (Fig. 5F–H and Supplementary Fig. S6B–S6E). We also observed a lesser damage to genitourinary tract (GJF; Supplementary Fig. S6A). These results indicate that the combination treatment was not toxic to normal tissues and in fact, silibinin showed radioprotective response in normal tissues.

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**Figure 6.** Combining silibinin with IR leads to reduced expression of cell proliferation and DNA repair markers and enhances apoptosis in DU145 xenograft. Tumor xenograft tissue samples were immunohistochemically analyzed for Ki67, pChk-2 (T68), pH2A.X, and TUNEL-positive cells as detailed in Materials and Methods. A, the representative pictograph (>400, magnifications) for positive brown-stained cells (dark color) for each of the markers are shown from control, IR, SB, and IR–SB groups. Quantitative data for Ki67 (B), pChk-2(T68) (C), pH2A.X(S139) (D), and apoptotic cells (E) from 5 to 6 mice in each group; $P < 0.01$ ($\ddagger$) and $P < 0.001$ ($\ddagger\ddagger$) compared with respective control. F, a model summarizing the radiosensitizing action of silibinin in prostate cancer (PCa) cells. Black dotted arrows represent pathways activated by IR in prostate cancer cells, bold arrows represent the action of silibinin (SB), and crosses represent IR-activated responses blocked by silibinin.
Silibinin-mediated radiosensitization of prostate tumor involvement inhibited of DNA repair and enhanced apoptosis

The immunohistochemical analysis of tumor samples showed that IR and/or silibinin reduced the immunostaining for Ki67 (Fig. 6A). IR or silibinin alone decreased Ki67-positive cells by 25% and 21%, respectively; however, their combination resulted in 54% (P < 0.001) decrease versus control, and 33% (P < 0.01) versus IR alone treatment (Fig. 6B), which supported the corresponding decrease in tumor burden. For the translational relevance of the in vitro observations of DNA repair signaling, tumors were analyzed for the pChk2 (Fig. 6A and C), which was increased by approximately 7-fold (P < 0.001) by IR treatment and that was reduced by the silibinin treatment to approximately 2-fold (P < 0.001) when compared with control (Fig. 6C). Next, the DNA DSBs were analyzed by immunostaining of pH2A.X (S139) (Fig. 6A and B), which was increased to 4% as compared with 1% in control, whereas in combination with silibinin, as anticipated, it increased to 13% (P < 0.001), suggesting that the mechanism of silibinin-mediated radiosensitization involved reduced DSB repair signaling (Fig. 6D). By TUNEL staining of tumor tissue (Fig. 6E), no significant apoptosis induction was observed with radiation or silibinin alone whereas their combination increased apoptotic cells by 10-fold (P < 0.001) from control and 5-fold (P < 0.001) from IR treatment (Fig. 6E).

Discussion

Radiotherapy is one of the principal and affordable treatment choices for locally or regionally advanced prostate cancer (19). However, development of radioresistance in these cells delimits its effectiveness in patients. With an aim to strengthen therapeutic outcomes, radiotherapy is often used in combination with drugs that are either cytotoxic or can radiosensitize or both (20, 21). These agents help in achieving the required remission at a much lower dose of radiation, thereby reducing the damage to normal tissues, which is a very germane issue in cancer treatment. However, currently, there is barely any radiosensitizer that has been successful in clinics.

In this study, we demonstrated the radiosensitizing effects of silibinin in prostate cancer cells. Silibinin enhanced the efficacy of radiotherapy in prostate cancer via (i) enhancing and prolonging the G2–M cell-cycle arrest induced by IR, (ii) augmenting ROS levels and sustaining high level of oxidative stress, (iii) inhibiting IR-induced prosurvival signaling and antiapoptotic pathways, (iv) inhibiting IR-induced DNA repair signaling in prostate cancer cells and tumors. These mechanisms eventually contributed to decreased cell growth, clonogenicity, and increased cell death; subsequently improving radiotherapeutic response. In addition, silibinin also helped in countering IR-induced toxicity in normal tissues.

One of the mechanisms for radiosensitizing effect of a drug could be its ability to affect cell-cycle progression especially by blocking it in G2–M phase of the cell cycle (22). Our study found that silibinin could enhance IR-induced G2–M arrest and also prolongs the duration of the arrest. This is of high significance in fractionated radiotherapy, G2–M phase being the most radiosensitive phase in the cell cycle, arresting a maximal population of cells in this phase would subsequently sensitize them to next cycle of radiation and enhance cell killing (23).

Other than cell-cycle perturbations, radiation-induced damage is essentially orchestrated via the production of ROS, which targets macromolecules, causing severe damage leading to cell death (24). Radiation-induced ROS levels peak within minutes of exposure and after the peak, it maintains a medium level of ROS lasting for days after irradiation (25). Unlike in normal cells, this moderate level of ROS is well tolerated by cancer cells, as these cells manipulate their redox system and generally have high levels of antioxidant enzymes to counter these conditions (26, 27). It has also been shown that in cancer stem cells, persistent low levels of ROS could eventually help in development of radioresistance (28). We demonstrated that addition of silibinin along with IR, can dramatically increase the level of ROS which, when retained for a longer duration, overpowers the robust antioxidant defense in cancer cells and drives the cells to death. Thus, silibinin enhances the ionizing radiation (IR)-induced oxidative stress to a level where it does not contribute to development of resistance, instead maintains it high and persistent enough to induce cell death. Another significant finding was that silibinin showed this pro-oxidant behavior exclusively in cancer cells, but not in normal cells. This biased behavior in modulating the redox status of cancer cells is a valuable asset for a radiosensitizer. This finding could also explain the disparity in response observed in clonogenic assay with HEK-293 cells when compared with other cancer cells.

Studies done in the past looking at the mechanisms of radioresistance in prostate cancer have pointed out that the upregulation of prosurvival signaling and the tipping of the balance toward antiapoptosis, compromises with the therapeutic efficacy of IR. The overexpression of Bcl-2 enhances radiation resistance in prostate cancer and other cancer cells (29, 30) and the suppression of which could overcome resistance (16). We demonstrate that silibinin could downregulate IR-induced survival signaling. Silibinin downregulated IR-induced Bcl-2 and survivin expression in DU145 and PC-3 cells, thereby maneuvering the cells toward apoptosis. Most cancer cells boast of a robust DNA repair system, which also contributes to acquiring a radioresistant phenotype. Thus, DNA repair proteins are now regarded as key targets for radiosensitization (17, 31). Our study found that silibinin could downregulate the repair process by inhibiting the expression of ATM as well as other downstream effectors, including Chk1 and Chk2.

One of the major players, which is involved in both DNA repair and upregulation of prosurvival signaling, is EGFR (7, 8). Most of the earlier studies linking EGFR and radioresistance focused on the receptor signaling induced by EGFR after ligand-independent activation in response to IR (32). But recent literature suggests that in response to IR, EGFR could contribute directly to development of radioresistance by its role in the nuclear compartment where it regulates DNA repair along with DNA-PK, which is a key regulator of NHEJ (7, 8, 33, 34). We found that silibinin treatment blocked nuclear translocation of EGFR. Silibinin also modulated the distribution of DNA-PK, restraining it from entering the nucleus to carry out its function, in concurrence with EGFR. The exclusion of EGFR and DNA-PK from the nucleus prevented the repair of DNA lesions, as shown by significantly enhanced number of γ-H2A.X foci. This is the first report of a phytochemical-modulating DNA repair by blocking the nuclear translocation of EGFR.

Many radiosensitizers though seem effective in vitro, but do not work under in vivo conditions and also have problems associated with toxicity. IR and silibinin combination strongly
decreased tumor burden and also reduced Ki67-positive cells in these tumors. We also observed intense staining for γ-H2AX and inhibition in Chk2 phosphorylation, suggesting inhibition of DNA repair signaling induced by IR. From our previous prostate cancer xenograft study (11), we know that the selected oral dose of silibinin used for the combination with IR is nontoxic. We observed significant reduction in the body weight and diet consumption in the IR alone group; however, the combination showed substantial improvement in these parameters. We also found that systemic toxicity of IR, mainly on the hematopoietic system was greatly reduced by silibinin treatment.

In conclusion, we, for the first time, report that silibinin functions as a potent radiosensitizer in human prostate cancer cells, and more importantly it offers substantial protection to the normal tissues from unwarranted IR toxicity. Silibinin targets multiple pathways, including DNA repair signaling involving nuclear translocation of EGFR, which are implicated in development of radioresistance (Fig. 6f). Earlier studies done with sili- binin showed that a concentration of up to 100 μmol/L could be achieved in blood plasma in mouse (35) as well as in humans (36), which signifies that the dose used in study (25 μmol/L) could be realized in patients undergoing radiotherapy for prostate cancer, and thus underlines the translational significance of this study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D.K. Nambiar, R. Agarwal, R.P. Singh
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.K. Nambiar, G. Deep, R. Agarwal, R.P. Singh
Writing, review, and/or revision of the manuscript: D.K. Nambiar, G. Deep, R. Agarwal, R.P. Singh
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Agarwal, R.P. Singh
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References


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