Inhibition of P-TEFb by DRB Suppresses SIRT1/CK2α Pathway and Enhances Radiosensitivity of Human Cancer Cells

ZHI-QIANG WANG, CASEY L. JOHNSON, AMIT KUMAR, DAVID P. MOLKENTINE, JESSICA M. MOLKENTINE, TATIANA RABIN, KATHRYN A. MASON, LUKA MILAS and UMA RAJU

Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, U.S.A.

Abstract. *Background: Positive transcription elongation factor-b (P-TEFb) is a complex containing CDK9 and a cyclin (T1, T2 or K). The effect of inhibition of P-TEFb by 5,6-dichloro-l-β-D-ribofuranosyl benzimidazole (DRB) on cell radiosensitivity and the underlying mechanisms were investigated. Materials and Methods: Six human cancer cell lines were subjected to ^{3}H-uridine incorporation, cell viability and clonogenic cell survival assays; cell-cycle redistribution and apoptosis assay; western blots and nuclear 53BP1 foci analysis after exposing the cells to DRB with/without γ-radiation. Results: DRB suppressed colony formation and enhanced radiosensitivity of all cell lines. DRB caused a further increase in radiation-induced apoptosis and cell-cycle redistribution depending on p53 status. DRB prolonged the presence of radiation-induced nuclear p53 binding protein-1 (53BP1) foci and suppressed the expression of sirtuin-1 (SIRT1) and casein kinase 2-alpha (CK2α), suggesting an inhibition of DNA repair processes. Conclusion: Our findings indicate that DRB has the potential to increase the efficacy of radiotherapy and warrants further investigation using in vivo tumor models.*

Cyclin-dependent kinases (CDKs) are critical regulators of cell-cycle progression and they are regulated by their modulators, primarily cyclin partners and specific endogenous inhibitors (1, 2). Besides being involved in cell-cycle control, some CDKs participate in transcriptional regulation (3, 4). Among them, CDK9 has been a focus in recent years. Positive transcription elongation factor-b (P-TEFb) is a complex containing CDK9 as its catalytic subunit and a cyclin as a regulatory subunit (3). Cyclin T1, T2 or K binds to CDK9 and facilitates the phosphorylation of RNA polymerase II (RNA pol II) to promote transcriptional elongation (4). The human RNA pol II carboxyl terminal domain (CTD) contains 52 tandem repeats of the consensus hepta-peptide sequence N-Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7-C. The CTD at serine 2 and serine 5 of the large subunit of RNA pol II is regulated by phosphorylation mediated by CDKs, including CDK9 (5). The expression of CDKs and their modulators frequently exhibit abnormal activities in many malignancies (6, 7). Inhibitors of CDKs have been of great interest in the treatment of cancer for the past several years (1, 2). The first small-molecule CDK inhibitor to be tested in clinical trials, flavopiridol, is a broad-spectrum inhibitor of CDKs and other protein kinases, such as protein kinase C and epidermal growth factor receptor; however, flavopiridol exerts the strongest inhibitory activity on all CDKs (8). Flavopiridol has been shown to inhibit CDK9 activity, leading to reduction in transcription of genes associated with induction of apoptosis (9, 10). Other CDK inhibitors such as SNS-032 (BMS-387032), R-roscovitine (CYC-202) and AT7519, are also associated with inhibition of RNA polymerase II activity (11-13).

We previously reported that flavopiridol potently enhances the radiosensitivity of mouse and human cancer cells and the underlying mechanisms include inhibition of DNA repair, induction of cell-cycle redistribution and apoptosis (14). However, as a broad-spectrum kinase inhibitor, flavopiridol blocks the activity of all CDKs, exhibiting a high level of toxicity (15). Thus, there is a need for a more specific CDK inhibitor that may have the potential to increase cancer cell response to radiation therapy with less toxicity.

An inhibitor of CDK9, 5,6-dichloro-l-β-D-ribofuranosyl benzimidazole (DRB) inhibits P-TEFb kinase activity and prevents phosphorylation of the CTD tail of RNA pol II and hence blocks the transition from transcription initiation to transcription elongation (16-18). Inhibition of transcription

Correspondence to: Uma Raju, Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030, U.S.A. E-mail: uraju@mdanderson.org

Key Words: DRB, radiosensitivity, RNA polymerase II, CDK9, SIRT1, CK2α, DNA repair.

by DRB has shown to block radiation-induced expression of a few DNA repair proteins such as excision repair cross-complementation group 1 (ERCC1) and X-ray repair cross-complementing protein 1 (XRCC1) (19). There are reports showing that DRB induces accumulation of p53 and when applied with radiation, it also enhances p53 accumulation in the nucleus (20). Because DRB has the potential to alter the expression of DNA-repair proteins and p53, the present study investigated whether DRB enhances cell radiosensitivity, and attempted to delineate the underlying mechanisms.

Materials and Methods

Cell culture. Three human head and neck squamous cell carcinoma (HNSCC) cell lines (HN5, HN30, and FaDu) and three human non-small cell lung cancer (NSCLC) cell lines (H460, H1299 and A549) were used in this study. H460, H1299 and A549 and FaDu were obtained from the American Type Culture Collection, Manassas, VA, USA. HN5 and HN30 were obtained from the University of Texas M.D. Anderson Cancer Center Core facility and grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Mediatech Inc. Herndon, VA, USA) or RPMI medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) and 10,000 units/ml penicillin-streptomycin (Gibco Life Technologies). All cell lines were validated by short tandem repeat profiling by the Characterized Cell Line core at the University of Texas M.D. Anderson Cancer Center (supported by CA016672).

DRB. DRB was obtained as a pure powder (Sigma-Aldrich) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and presented to the cells in culture with a final DMSO concentration of <0.001%.

3-[4,5-Dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were plated in a 96-well plate, and the next day, they were treated with different concentrations of DRB (10, 50, 100 μM). After one day, cells were stained with MTT (200 μg/ml; Sigma-Aldrich) and then lysed in ethanol:DMSO mixture (1:1). The absorbance was read at 540 nm using a 96-well plate reader.

^{3}H-Uridine incorporation. Cells were plated in 60 mm plates, and the next day, they were treated with different concentrations of DRB (10, 50, 100 μM). After 22 h, cells were labeled for 2 h with 5 μCi/ml of ^{3}H-uridine (Perkin-Elmer, Santa Clara, CA, USA). Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in 100 μl of western stock extraction buffer (1 M Hepes, 5 M NaCl, 0.5 M EDTA, pH8 and 0.1 M EGTA, pH 7). The solution was mixed thoroughly, then 5 μl was removed for protein assay. An additional 900 μl extraction buffer was added to the remaining 95 μl. Ice-cold 100% trichloroacetic acid solution (Sigma-Aldrich) was added to these lysates to a final concentration of 5% (v/v). Cell lysates were vortexed immediately and kept on ice for 30 min, and precipitates were collected by vacuum-assisted filtration on glass microfiber filter disks (Whatman, 2.4 cm). Filters were allowed to dry in air, and radioactivity was determined by immersing filters in 6 ml of scintillation fluid (Liquiscint; National Diagnostics, Atlanta, GA, USA) followed by liquid scintillation counting. Radioactivity was normalized to protein in the lysates, which was determined with the Pierce BCA protein assay kit (Thermo Scientific, Pittsburgh PA, USA).

Clonogenic cell survival assay. Cells in culture were exposed to DRB (10, 50, or 100 μM) for 6 or 24 h, after which they were irradiated with graded doses (2, 4, or 6 Gy) of γ-rays using a ^{137}Cs source (3.7 Gy/min). At the end of the treatments, the cells were assayed for colony-forming ability by replating them in specified numbers in 60-mm dishes in drug-free medium. After 12 days of incubation, the cells were stained with 0.5% crystal violet in methanol, and colonies with more than 50 cells were counted under a dissection microscope. Radiation survival curves were plotted after normalizing for the cytotoxicity induced by DRB alone. Clonogenic survival curves were constructed by fitting the average survival levels using least squares regression by the linear quadratic model.

Apoptosis and cell-cycle distribution. Cells were plated in 100-mm dishes and treated with DRB (100 μM), γ-radiation, or both, where the cells were treated with DRB for 24 h, then exposed to radiation. After irradiation, DRB was removed by changing to new culture media. Cells were subjected to terminal deoxy-nucleotidyltransferase (TdT) dUTP nickend labeling (TUNEL) assay. An Apo-Direct kit (BD Pharmingen, San Jose, CA, USA) was used following the manufacturer's protocol, including that for staining with propidium iodide for cell-cycle analysis. Cells were analyzed by flow cytometry to quantify the numbers of apoptotic cells and the distribution of cells throughout the cell cycle.

Immunocytochemical analysis. Cells were grown on coverslips placed in 35-mm dishes for 48 h then treated with DRB, radiation, or both. After an incubation period of 30 min, 4 h, 16 h or 24 h, the cells were washed with PBS, and then fixed in 1% paraformaldehyde first and then in 70% ethanol and processed for immunofluorescent staining. Then the cells were exposed to 1% Igepal (Sigma-Aldrich). After washing with PBS, cells were exposed to a blocking buffer containing 5% bovine serum albumin in PBS before incubation with primary antibody. The primary antibody (1:300 dilution) used was rabbit antibody to 53BP1 (Cell Signaling Technology, Danvers, MA, USA). After overnight incubation in primary antibody, cells were washed with PBS and then exposed to Cy3 (red)-conjugated anti-rabbit immunoglobulin (1:500 dilution; Jackson Immunoresearch Labs Inc., West Grove, PA, USA). After washing with PBS, cells were exposed to 4',6-diamidino-2-phenylindole (DAPI) (1:1000 dilution; Sigma-Aldrich) for DNA staining.

Western blot analysis. Cells were grown in 10-cm dishes and exposed to DRB, radiation or both and at specified time points cells were collected and subjected to Western blot analysis as described (14). All primary antibodies were obtained from Cell Signaling Technology and the secondary antibodies were from Jackson Immunoresearch Labs Inc. The immunoreaction was visualized using enhanced chemiluminescence-Plus detection system (Amersham, Arlington Heights, IL).

Statistical analytical methods. Student's *t*-tests were used to determine statistical differences between the various experimental groups; $p<0.05$ was considered to be significant.

Results

DRB partially inhibited ^{3}H-uridine incorporation. To determine the ability of DRB in blocking RNA pol II activity, incorporation of ^{3}H-uridine into RNA was assessed as a measure of transcription. HN5, HN30 and H460 cells were treated with different concentrations (10, 50, 100 μM) of DRB for 24 h. During the last 2 h, cells were labeled with 5 μCi/ml of ^{3}H-uridine. Cell lysates were collected, precipitated by TCA solution and then transferred to the microfiber filter disks. The radioactivity was determined by liquid scintillation counting and then normalized to protein level in the lysates. Significant reduction in ^{3}H-uridine incorporation was observed at doses of 50 μM and 100 μM (Figure 1A).

DRB reduced cell viability and clonogenicity. The dose-dependent cytotoxicity of DRB alone was determined by the MTT assay (20). Cells were incubated in the presence of DRB, ranging from 10 to 100 μM, for 24 h and subjected to MTT assay. DRB markedly reduced cell survival at 50 μM and the percentage survival ranged from 47.5 to 72.6%. A further reduction in cell survival was observed in some cell lines at 100 μM DRB (Figure 1B). To determine whether the clonogenicity of cells was reduced by DRB, cells were exposed to DRB for 24 h and replated for colony formation. After 12 days in drug-free medium, colonies were stained and counted. DRB reduced the number of colonies in a cell type-dependent manner (Figure 1C).

DRB enhanced radiosensitivity of cells. To determine the radiosensitizing effect of DRB, clonogenic cell survival was assayed after exposing the cells to DRB (50 or 100 μM) for 6 or 24 h and then treating them with 2-6 Gy of γ-radiation. The cells were then plated and incubated for 12 days to determine the ability for colony formation. Figure 2 shows radiation dose–response curves without and with DRB (100 μM) treatment given for 24 h before irradiation; lower doses of DRB and shorter treatments had either no effect or lesser effect than those shown in Figure 2. The curves representing the effect of DRB-plus-radiation were normalized, *i.e.* the colony number after drug alone was set at a level of 1 (100% survival). Radiation-alone caused a dose-dependent reduction in cell survival. Treatment with DRB enhanced the radiation-induced cell killing, the extent of which depended on its concentration: the effect increased as the dose of DRB increased. Enhancement factors (EFs) were calculated at the surviving fraction of 0.1 by dividing the effect of the radiation dose of the control curve with that of the corresponding curves for treatment with DRB-plus-radiation. DRB treatment (100 μM) for 6 h increased the radiosensitivity of H460 cells (EF=1.23), but did not affect the radiosensitivity of H1299, HN5 and HN30 cells (Table I). DRB treatment at 10 μM for 24 h did not affect the radiosensitivity of HN5, HN30 and

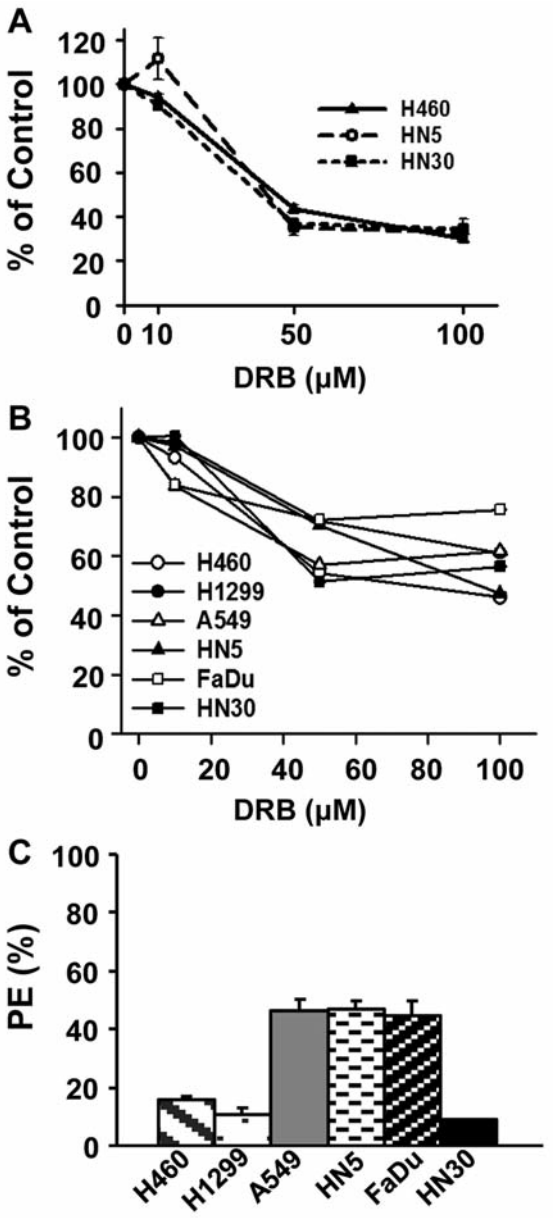

Figure 1. *A: 5, 6-dichloro-l-β-D-ribofuranosyl benzimidazole (DRB) inhibited 3H-uridine incorporation. Cells were exposed to DRB (10-100 μM) for 24 h. During the last 2 h, 3H-uridine (10 mCi/ml) was added to cells. Cells were washed and lysed with extraction buffer, then precipitated with trichloroacetic acid solution. Radioactivity of the cells was obtained by liquid scintillation counting and then normalized to the protein level in the lysates. B: DRB suppressed cell viability. Cells were treated with DRB (10-100 μM) for 24 h in 96-well plates. The cells were then stained with MTT reagent and lysed, and the absorbence was read in a plate reader using a 540-nm filter. Surviving cells were calculated as a percentage that of the control. DRB reduced the cell viability in a dose-dependent manner; the 100 μM dose was chosen for further investigation. C: DRB blocked the colony-forming ability of cells. Colony-forming ability of cells was determined by plating known number of cells after exposing them to DRB (100 μM, 24 h). After incubating the cells in DRB-free culture medium for 10-4 days, colonies with more than 50 cells were stained and counted. Colonies numbers are plotted as a percentage of the plating efficiency (PE) of untreated control cells.*

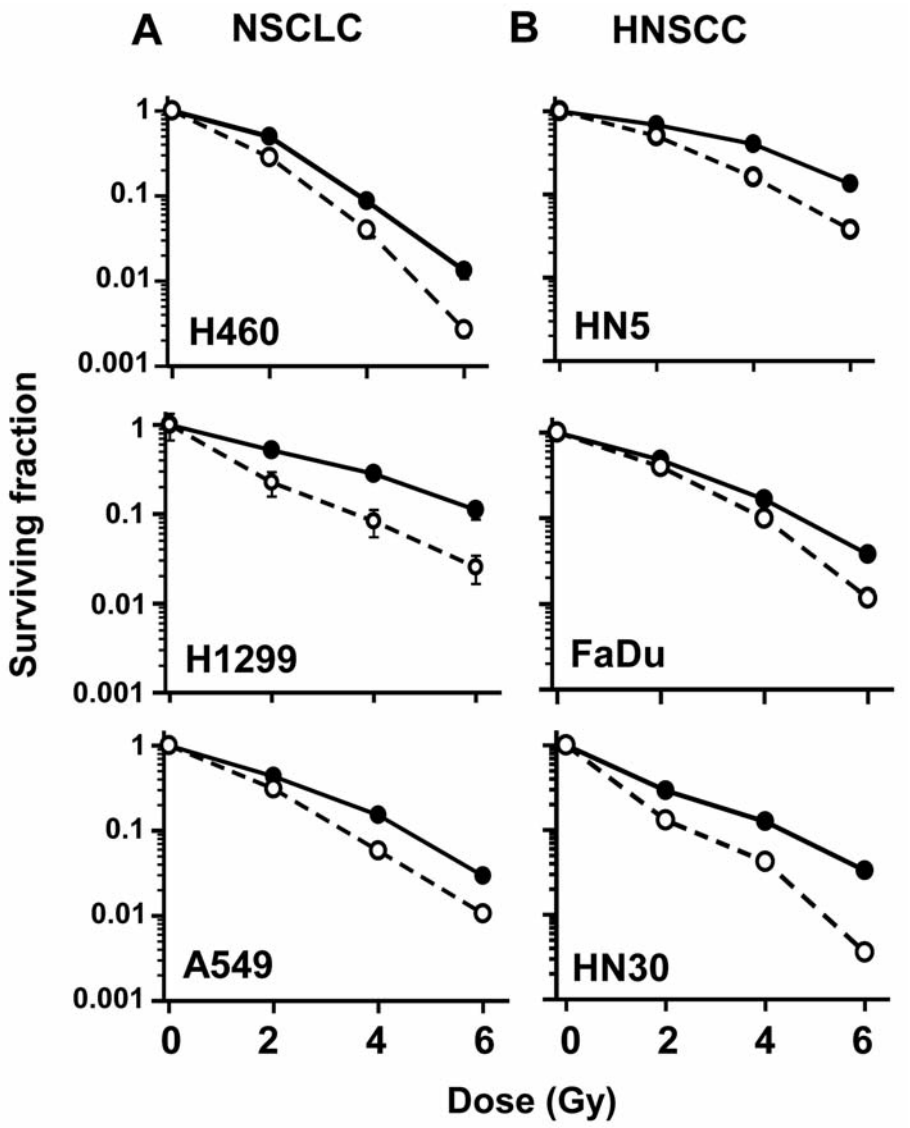

Figure 2. *5, 6-dichloro-l-β-D-ribofuranosyl benzimidazole (DRB) enhanced cell radiosensitivity. The cell radiosensitivity was determined by plating a known number of cells after exposing them to radiation (2, 4 or 6 Gy) without or with DRB pretreatment (100 μM, 24 h). After incubating the cells in DRB-free culture medium for 10-14 days, colonies with more than 50 cells were stained and counted. The survival curves were constructed with normalized values for the cytotoxicity induced by DRB alone. Values shown are the means±SE for three independent experiments.*

FaDu cells (data not shown). DRB treatment at 50 μM affected the radiosensitivity of H460, H1299 and HN5 cells (EFs of 1.56, 1.45 and 1.43, respectively), but had no effect on HN30 cells. Treatment of 100 μM DRB for 24 h enhanced the radiosensitivity of all these cell lines, including A549 and FaDu (Table I). DRB did not increase the radiosensitivity of cells when given 7 h after irradiation (data not shown).

DRB induced apoptosis in a cell type-dependent manner. To determine whether DRB induced apoptosis in tumor cells, the cells were subjected to the TUNEL assay. Untreated H460 and HN5 cells underwent apoptosis, 1.4% and 2%, respectively. Radiation alone at a dose of 4 Gy did not induce any significant amount of apoptosis in H460 and HN5 cells when analyzed one day after irradiation (Figure 3). DRB-treated H460 cells exhibited 82.4% apoptosis and but DRB had a minimal effect on HN5 cells (4.1% apoptosis). The combination of DRB and 4 Gy further increased the number of apoptotic H460 cells (92.9% apoptosis), but led to only a small increase in apoptosis in HN5 cells (10.9%). These observation indicate that DRB rendered H460 tumor cells more susceptible to radiation-induced apoptosis, but not HN5 cells.

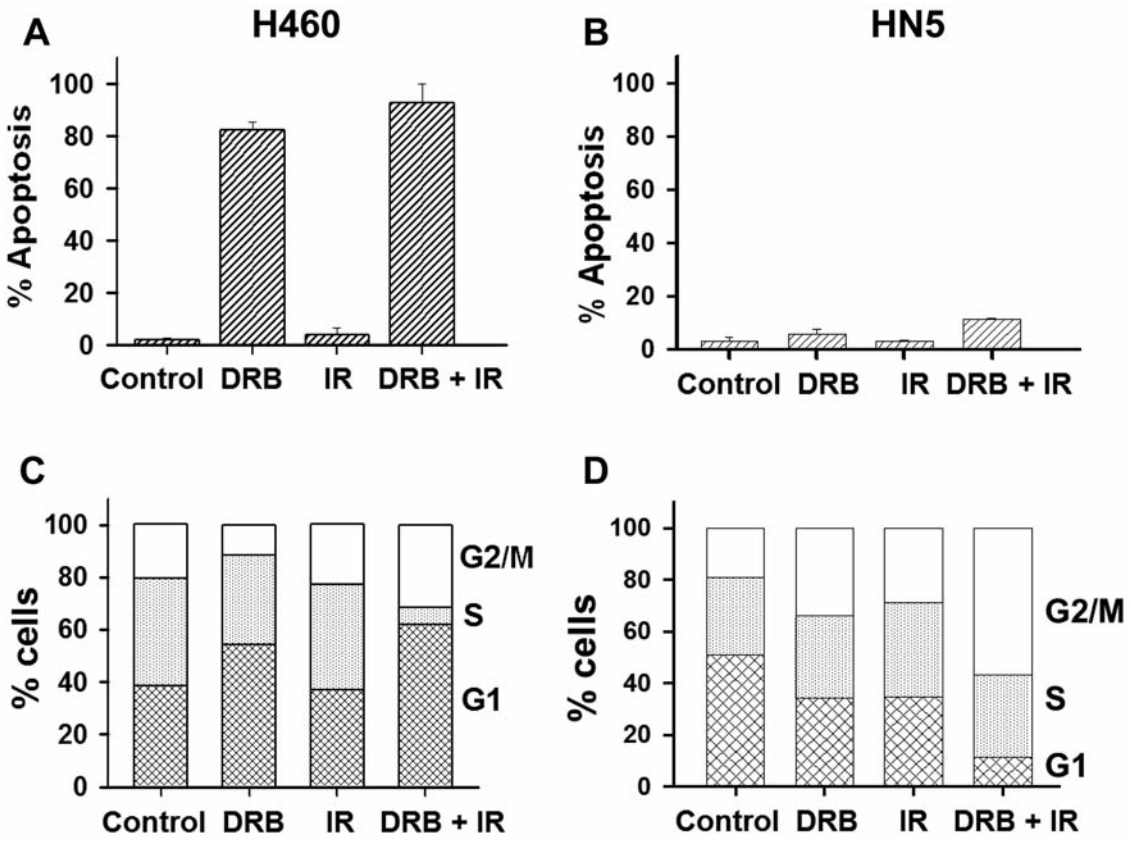

Figure 3. *Induction of apoptosis and cell-cycle redistribution by 5, 6-dichloro-l-β-D-ribofuranosyl benzimidazole (DRB) and radiation treatments. A and B: Cells were exposed to 100 μM DRB for 24 h, 4 Gy of γ-radiation (IR), or both. Then the cells were processed for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (Apo-Direct Kit, Pharmingen, San Diego, CA, USA) and apoptotic cells were quantitated by flow cytometry analysis. C and D: Cells were treated with 100 μM DRB for 24 h, γ-radiation, or both. They were then trypsinized and fixed in 70% ethanol. After washing, the cells were exposed to propidium iodide/RNase solution. Cells were analyzed by flow cytometry for distribution of cells throughout the cell cycle.*

DRB prolonged the presence of radiation-induced nuclear 53BP1 foci. To investigate whether the radioenhancement induced by DRB involved inhibition of DNA repair, cells were subjected to staining of 53BP1, a marker of DNA double-strand breaks. At different time points after treatment with DRB (100 μM, 24 h), radiation (2 Gy), or both, cells were processed for immunocytochemical analysis. Figure 4A illustrates representative nuclei of cells under each treatment condition at 30 min, 4 h, 16 h or 24 h after treatment. The immunoreaction with 53BP1 was visualized using a Cy3-conjugated secondary antibody. Quantification of 53BP1 foci showed that radiation exposure induced the appearance of 53BP1 foci in more than 80% of nuclei in H460 cells and the presence of DRB did not have any significant effect at the 30 min time point (Figure 4B). However, by 4 h after irradiation, the number of nuclei with 53BP1 foci had decreased to about 60% in radiation-only-treated cells, which further decreased to less than 20% at the 24-h time point. When the cells were exposed to DRB first and then to radiation, the presence of 53BP1 foci in the nuclei was prolonged and at the 24-h time point, 40% of nuclei were positive for 53BP1 foci (Figure 4C). Similar data were obtained for HN5 cells (data not shown).

DRB and radiation altered the expression levels of apoptotic and cell-cycle proteins. Western blot analysis was performed to determine the effects of DRB with/without radiation on some of the proteins that are known to participate in apoptotic, cell-cycle and DNA repair pathways. Figure 5A shows the effect of DRB-alone on H460 cells. Cleaved caspase-3 was induced after 16 to 24 h of DRB treatment. Accumulation of p53 was apparent after 2 to 24 h of DRB treatment. Cyclin-B1 expression was reduced by DRB treatment at 4 h and by 16 h it was markedly reduced. As shown in Figure 5B, radiation (6 Gy) and DRB induced the accumulation of p53 protein that was further increased after

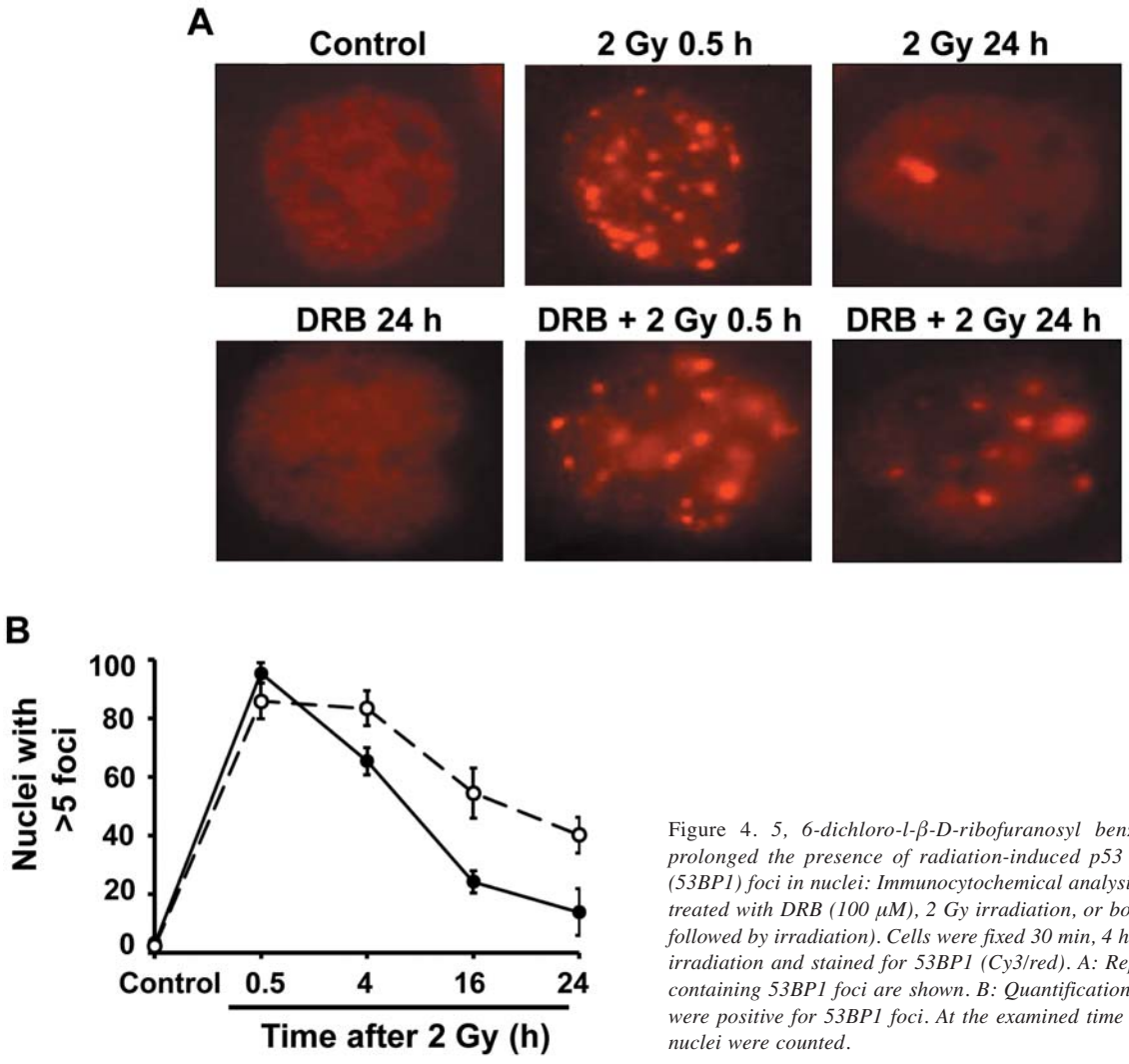

Figure 4. *5, 6-dichloro-l-β-D-ribofuranosyl benzimidazole (DRB) prolonged the presence of radiation-induced p53 binding protein 1 (53BP1) foci in nuclei: Immunocytochemical analysis. H460 cells were treated with DRB (100 μM), 2 Gy irradiation, or both (DRB treatment followed by irradiation). Cells were fixed 30 min, 4 h, 16 h or 24 h after irradiation and stained for 53BP1 (Cy3/red). A: Representative nuclei containing 53BP1 foci are shown. B: Quantification of cell nuclei that were positive for 53BP1 foci. At the examined time point, at least 100 nuclei were counted.*

the combined treatment of these two agents. BCL2 was suppressed and cleaved caspase-3 was increased after the combination treatment.

In HN5 cells, there was a minimal effect on cleaved caspase-3 and no effect on p53 levels after DRB treatment (Figure 5C). Although radiation slightly increased the level of p53 protein, the presence of DRB blocked this slight increase (Figure 5D). BCL2 was suppressed by DRB and by combination treatment. However, there was no effect on caspase-3.

DRB and radiation altered the expression levels of RNA pol II, SIRT1 and CK2α in both H460 and HN5 cells. Because P-TEFb phosphorylates RNA pol II, we tested the effect of DRB with and without radiation on the level of RNA pol II. Additionally, we tested the effect of DRB and radiation on SIRT1 and the associated protein CK2α, as both are modulated by cellular myelocytomatosis (*c-MYC*) oncogene that is regulated by P-TEFb. Cells were exposed to DRB, radiation or both and at specified time points cell lysates were subjected to western blot analysis. As shown in Figure 6A, irradiation of H460 cells did not have any profound effect on RNA pol II, but DRB markedly reduced it. In both H460 and HN5 cells, DRB suppressed the levels of SIRT1, as well as CK2α. Combined treatment with DRB and radiation resulted in reduced levels of these two proteins.

Discussion

Radiation treatment is the first-line treatment for most HNSCCs and NSCLCs, and resistance to radiation therapy is associated with the repair capacity of cells, among other cellular functions (21). Many radiosensitizers have been reported to interfere with the DNA-repair processes by

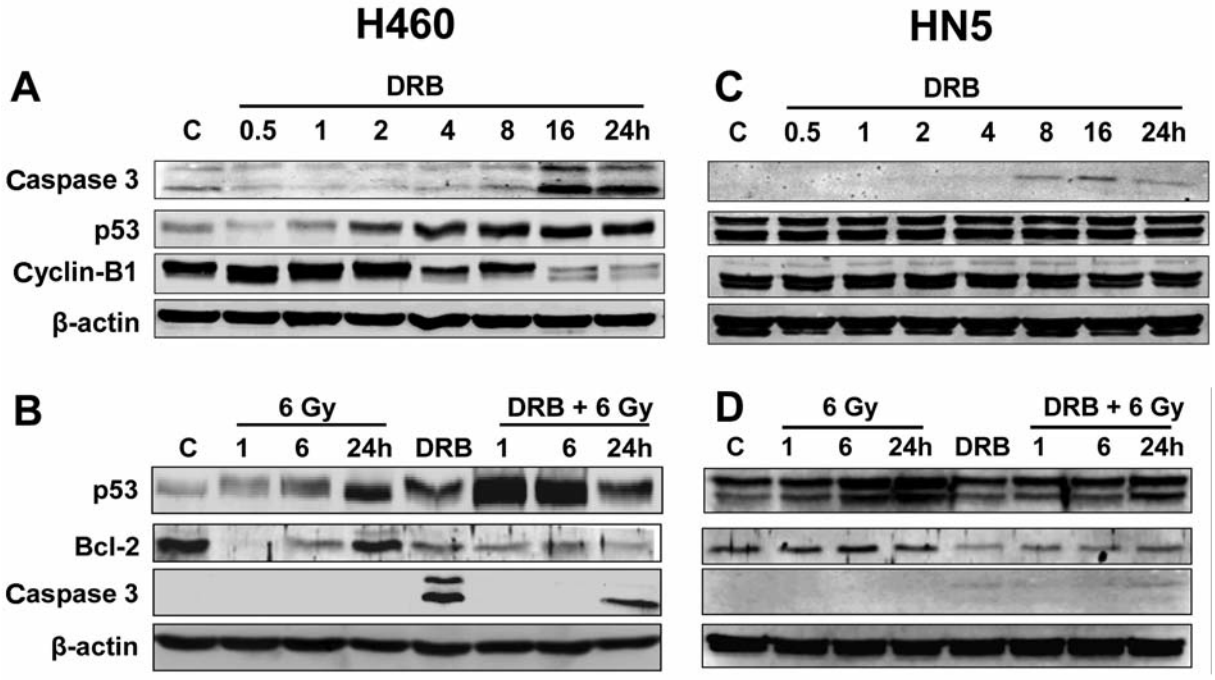

Figure 5. *Effect of 5, 6-dichloro-l-β-D-ribofuranosyl benzimidazole (DRB) on proteins associated with apoptosis and cell cycle. Cells were exposed to DRB with/without radiation (6 Gy). At specified time points, cell lysates were prepared and subjected to western blot analysis. Actin served as a loading control. Blots shown are representative of two independent experiments.*

which they increase radiation-induced cell killing (14, 22, 23). Our study showed that DRB effectively augmented the radiosensitivity of all six cancer cell lines tested and the underlying mechanisms appeared to be multiple and cell line-specific. Consistent with previous reports (20, 24), DRB induced accumulation of p53 protein and when combined with radiation, a synergistic effect on p53 was observed in H460 cells that harbor wild-type p53 protein. In HN5 cells, which have mutant p53 protein, DRB did not have any effect on the p53 level. Thus, the effect of DRB on p53 was dependent on p53 status. However, the effect of DRB on radiosensitivity of cells did not depend on the *p53* status. Cells carrying either wild-type *p53* (H460, A549 and HN30) or mutant/null *p53* (HN5, FaDu and H1299) showed increased cell radiosensitivity after exposure to DRB. Interestingly, DRB induced apoptosis only of *p53* wild-type cell line, H460, and not in the *p53*-mutant cell line, HN5.

These data suggest that induction of apoptosis by DRB may depend on functional p53 and may play a role in cellular response to radiation in H460 cells and not in HN5 cells. Involvement of p53 in the cancer cell response to radiotherapy has been well established (25). Alterations in cell-cycle distribution by DRB and radiation were also dependent on p53 status, where *p53* wild-type cells accumulated in the G_1 phase after treatments, HN5 cells with mutant *p53* accumulated in the G_2/M phase of the cell cycle that has been reported to be a relatively more radiosensitive phase of the cell cycle (26).

Table I. *Effect of 5,6-dichloro-l-p-D-ribofuranosyl benzimidazole (DRB) on cell radiosensitivity. Clonogenic cell survival assays were performed to assess the effect of DRB (50 or 100 μM, given for 6 or 24 h before irradiation.*

Cell lines	Dose (μM)	Time (h)	EF (at 0.1 SF)
H 460	50	24	1.26
H 460	100	6	1.16
H 460	100	24	1.26
H1299	50	24	1.36
H1299	100	6	0.96
H1299	100	24	1.86
A549	100	24	1.32
HN5	50	24	1.35
HN5	100	24	1.33
HN30	50	24	1.13
HN30	100	6	0.92
HN30	100	24	1.57
FaDu	100	24	1.2

EF: Enhancement factor; SF: survival fraction.

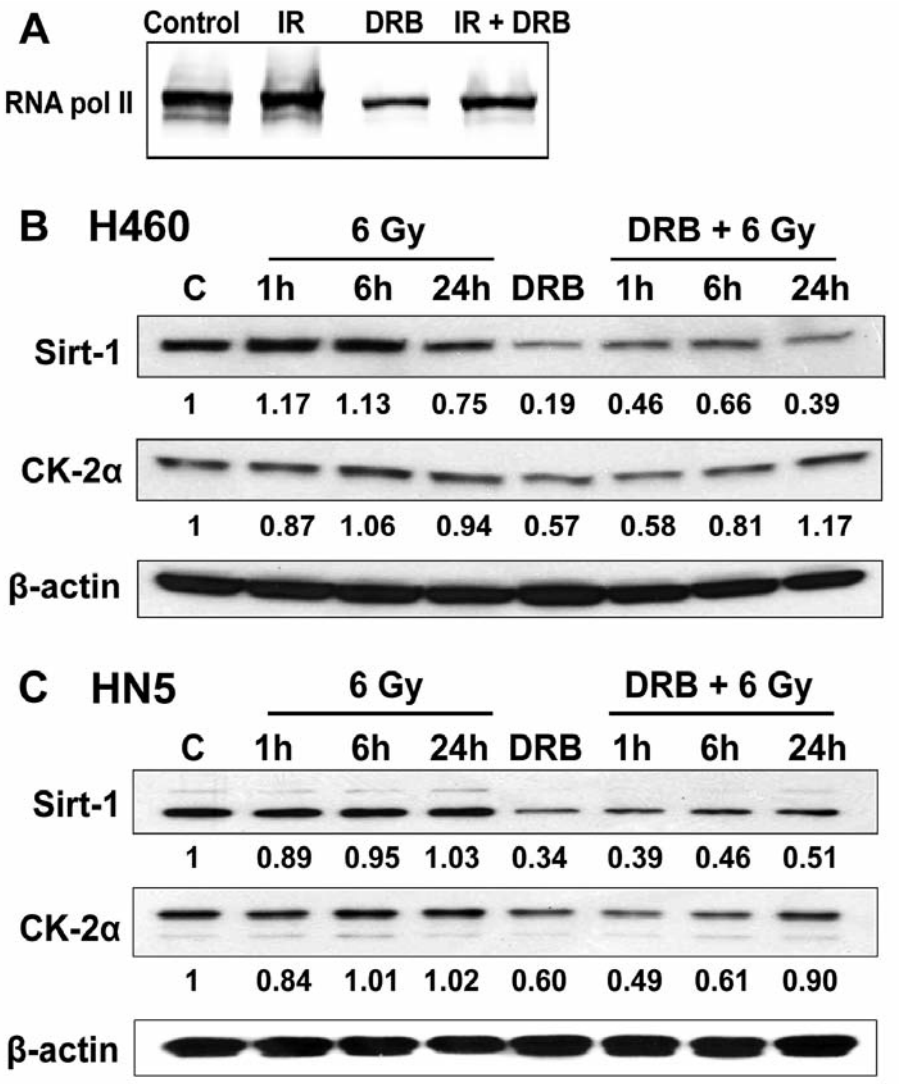

Figure 6. *5, 6-dichloro-l-β-D-ribofuranosyl benzimidazole (DRB) suppressed the expression of RNA polymerase II (RNA pol II), sirtuin 1 (SIRT1) and casein kinase 2-alpha (CK2α) proteins. Cells were exposed to DRB with/without radiation (6 Gy). At specified time points, cell lysates were prepared and subjected to western blot analysis. The numbers shown below the protein bands are band intensities relative to the untreated control protein set at a value of 1. Actin served as a loading control. Blots shown are representative of two independent experiments.*

Taken together, these data suggest that the underlying mechanisms of radiosensitizing effect of DRB are dependent on the cell line. In H460 cells, the mechanisms may include apoptosis and cell-cycle redistribution along with the inhibition of radiation-induced DNA repair; in HN5 cells, the mechanisms were primarily inhibition of DNA repair and cell-cycle redistribution.

Inhibition of P-TEFb by DRB resulted in reduction in SIRT1 and CK2α expression in both H460 and HN5 cells. CK2α is a regulator of SIRT1, and both CK2α and SIRT1 are implicated in cell-cycle progression and in mediating resistance to radiation (27, 28). Reports show that suppression of these two proteins enhances radiosensitivity of many cancer cell types, including hepatoma, glioblastoma and NSCLC cells (29-31). Thus, our data are consistent with the notion that suppression of CK2α and SIRT1 correlated with increased cell radiosensitivity.

In conclusion, DRB increased cancer cell radiosensitivity independently of p53 functional status. The underlying mechanisms may be multiple and cell line-specific. Inhibition of DNA repair, induction of apoptosis and cell-cycle redistribution and suppression of CK2α and SIRT1 are potential mediators of the effects of DRB. Thus, our findings indicate that DRB has the potential to increase the efficacy of radiotherapy for HNSCC and NSCLC and warrants further investigation using *in vivo* tumor models.

References

1 Shapiro GI: Cyclin-dependent kinase pathways as targets for cancer treatment. J Clin Oncol *24*: 1770-1783, 2006.

2 Sherr CJ and Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev *13*: 1501-1512, 1999.

3 Wang S and Fischer PM: Cyclin-dependent kinase 9: a key transcriptional regulator and potential drug target in oncology, virology and cardiology. Trends in Pharmacol Sci Rev *29*: 302-313, 2008.

4 Falco GD and Giordano A: CDK9: From basal transcription to cancer and AIDS. Cancer Biol Ther Rev *1*: 342-347, 2002.

5 Oelgeschlager T: Regulation of RNA polymerase II activity by CTD phosphorylation and cell cycle control. J Cell Phys *190*: 160-169, 2002.

6 MacLachlan TK, Sang N and Giordano A: Cyclins, cyclin-dependent kinases and CDK inhibitors: implications in cell cycle control and cancer. Crit Rev Eukaryot Gene Expr *5*: 127-156, 1995.

7 Zafonte BT, Hulit J, Amanatullah DF, Albanese C, Wang C, Rosen E, Reutens A, Sparano, JA, Lisanti MP and Pestell RG: Cell-cycle dysregulation in breast cancer therapies targeting the cell cycle. Frontiers Biosci *5*: D938-D961, 2000.

8 Senderowicz AM, Headlee D, Stinson SF, Lush RM, Kalil N, Villalba L, Hill K, Steinberg SM, Figg WD, Tompkins A, Arbuck SG, Sausville EA: Phase I trial of continuous infusion flavopiridol, a novel cyclin-dependent kinase inhibitor in patients with refractory neoplasms. J Clin Oncol *16*: 2986-2999, 1998.

9 Chao S, Fujinaga K, Marion JE, Taube R, Sausville EA, Senderowicz AM, Peterlin BM and Price DH: Flavopiridol inhibits P-TEFb and blocks HIV-1 replication. J Biol Chem *275*: 28345-28348, 2000.

10 Wang K, Hampson P, Hazeldine J, Krystof V, Strnad M, Pechan P and Janet M: Cyclin-dependent kinase 9 activity regulates neutrophil spontaneous apoptosis. PLoS ONE *7*: e30128, 2012.

11 MacCallum DE, Melville J, Frame S, Watt K, Anderson S, Gianella-Borradori A, Lane DP and Green SR: Seliciclib (CYC202, R-Roscovitine) induces cell death in multiple myeloma cells by inhibition of RNA polymerase II-dependent transcription and down-regulation of Mcl-1. Cancer Res *65*: 5399-407, 2005.

12 Chen R1, Wierda WG, Chubb S, Hawtin RE, Fox JA, Keating MJ, Gandhi V and Plunkett W: Mechanism of action of SNS-032, a novel cyclin-dependent kinase inhibitor, in chronic lymphocytic leukemia. Blood *113*: 4637-4645, 2009.

13 Santo L, Vallet S, Hideshima T, Cirstea D, Ikeda H, Pozzi S, Patel K, Okawa Y, Gorgun G, Perrone G, Calabrese E, Yule M, Squires M, Ladetto M, Boccadoro M, Richardson PG, Munshi NC, Anderson KC and Raje N: AT7519, A novel small molecule multi-cyclin-dependent kinase inhibitor, induces apoptosis in multiple myeloma *via* GSK-3β activation and RNA polymerase II inhibition. Oncogene *29*: 2325-2336, 2010.

14 Raju U, Nakata E, Mason KA, Ang KK and Milas L: Flavopiridol, a cyclin-dependent kinase inhibitor, enhances radiosensitivity of ovarian carcinoma cells. Cancer Res *63*: 3263-3267, 2003.

15 Hofmeister CC, Poi M, Bowers MA, Zhao W, Phelps MA, Benson DM, Kraut EH, Farag S, Efebera YA, Sexton J, Lin TS, Grever M and Byrd JC: A phase I trial of flavopiridol in relapsed multiple myeloma. Cancer Chemother Pharmacol *73*: 249-257, 2014.

16 Dreyer C and Hausen P: Inhibition of mammalian RNA polymerase by 5,6-dichloro-ribofuranosylbenzimidazole (DRB) and DRB triphosphate. Nucleic Acids Res *5*: 3325-3335, 1978.

17 Baumli S, Endicott JA and Johnson LN: Halogen bonds form the basis for selective P-TEFb inhibition by DRB. Chem Biol *17*: 931-936, 2010.

18 Gargano B, Amente S, Majello B and Lania L: P-TEFb is a crucial co-factor for Myc transactivation. Cell Cycle *6*: 2031-2037, 2007.

19 Yacoub A, Park JS, Qiao L, Dent P, Hagan MP: MAPK dependence of DNA damage repair: ionizing radiation and the induction of expression of the DNA repair genes XRCC1 and ERCC1 in DU145 human prostate carcinoma cells in a MEK1/2 dependent fashion. Int J Radiat Biol *77*: 1067-1078, 2001.

20 O'Hagan HM, Ljungman M: Phosphorylation and nuclear accumulation are distinct events contributing to the activation of p53. Mutat Res *546*: 7-15, 2004.

21 Li C, Brand TM, Iida M, Huang S, Armstrong EA, van der Kogel A and Wheeler DL: Human epidermal growth factor receptor 3 (HER3) blockade with U3-1287/AMG888 enhances the efficacy of radiation therapy in lung and head and neck carcinoma. Discov Med *16*: 79-92, 2013.

22 Raju U, Riesterer O, Wang ZQ, Molkentine DP, Molkentine JM, Johnson FM, Glisson B, Milas L and Ang KK: Dasatinib, a multi-kinase inhibitor increased radiation sensitivity by interfering with nuclear localization of epidermal growth factor receptor and by blocking DNA repair pathways. Radiother Oncol *105*: 241-249, 2012.

23 Matsumoto F, Valdecanas DN, Mason KA, Milas L, Ang KK and Raju U: The impact of timing of EGFR and IGF-1R inhibition for sensitizing head and neck cancer to radiation. Anticancer Res *32*: 3029-3035, 2012.

24 Blaydes JP, Craig AL, Wallace M, Ball HML, Traynor NJ, Gibbs NK and Hupp TR: Synergistic activation of p53-dependent transcription by two cooperating damage recognition pathways. Oncogene *19*: 3829-3839, 2000.

25 Beckta JM, Ahmad SF, Yang H, and Valerie K: Revisiting p53 for cancer-specific chemo- and radiotherapy. Cell Cycle *13*: 710-713, 2014.

26 Sinclair WK and Mortan RA: X-Ray sensitivity during the cell generation cycle of cultured Chinese hamster cells. Radiat Res *29*: 450-474, 1966.

27 Dixit D, Sharma V, Ghosh S, Mehta VS and Sen E: Inhibition of casein kinase-2 induces p53-dependent cell cycle arrest and sensitizes glioblastoma cells to tumor necrosis factor (TNFα)-induced apoptosis through SIRT1 inhibition. Cell Death Dis *3*: e271, 2012.

28 Nihal M, Ahmad N, and Wood GS: SIRT1 is up-regulated in cutaneous T-cell lymphoma, and its inhibition induces growth arrest and apoptosis. Cell Cycle *13*: 632-640, 2014.

29 Xie Y, Zhang J, Ye S, He M, Ren R, Yuan D and Shao C: SirT1 regulates radiosensitivity of hepatoma cells differently under normoxic and hypoxic conditions. Cancer Sci *103*: 1238-1244, 2012.

30 Kroonen J, Artesi M, Capraro V, Nguyen-Khac M, Willems M, Chakravarti A, Bours V and Robe PA: Casein kinase 2 inhibition modulates the DNA damage response but fails to radiosensitize malignant glioma cells. Int J Oncol *41*: 776-782, 2012.

31 Lin Y, Hung M, Lin C, Li J, Lee K, Li Y, Chen M, Chen J and Yang C: CK2 inhibitors enhance the radiosensitivity of human non-small cell lung cancer cells through inhibition of stat3 activation. Cancer Biother Radiopharm *26*: 381-388, 2011.

Received August 21, 2014
Revised September 25, 2014
Accepted September 30, 2014