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Radiosensitizing Effects of Plumbagin in Cervical Cancer Cells Is Through Modulation of Apoptotic Pathway

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Radiotherapy is the primary line of cancer treatment for cervical cancer and is known to induce cell death in tumors. Radiotherapy is however limited by the total dose that can be given without damaging normal tissue. Plumbagin, a naturally occurring naphthaquinone, has been reported to have free radical producing properties. Hence we hypothesized that plumbagin could also have properties that could modify effects of radiation on cervical cancer cells. Radiation in combination with plumbagin may thus have treatment augmenting effects. Results from our studies have shown that a lower dose of radiation in combination with plumbagin could induce apoptosis more effectively compared to a higher dose of radiation alone. Plumbagin in combination with 2 Gy of radiation was very effective in inducing apoptosis, when compared to a higher radiation dose of 10 Gy alone. This combination also showed a fivefold increase in the activation of caspase 3 in C33A cells. Activation of effector caspases confirms that the induction of apoptosis by irradiation and plumbagin involves caspase-dependent pathways. Expression of apoptotic regulatory molecules Bcl-2, Bax and Survivin was also modulated by plumbagin in combination with radiation. In summary, this study shows that a combination of plumbagin and radiation augmented cell growth inhibition compared to higher radiation dose alone, thus indicating that plumbagin may be a potential radiosensitizer acting through the induction of apoptosis.

Key words: plumbagin; radiotherapy; cervical cancer; apoptosis; caspases

INTRODUCTION

Cancer of the uterine cervix is the second leading cause of death from cancer in women worldwide [1]. Radiotherapy is an important treatment modality for cervical cancer, particularly for locally advanced tumors and is known to mainly induce cell death in tumors. Radiotherapy is limited by the total dose that can be given without damaging normal tissue. Any compound that increases the therapeutic efficacy of radiation without dose escalation (that is higher treatment response at lower doses) will therefore be beneficial for cancer treatment. Hence the study of such biological response modifiers is a prime requirement in cancer therapeutics. Quinones represent the largest class of quinoid compounds, which are widely distributed in nature. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a naturally occurring naphthoquinone derivative from plants belonging to Plumbaginaceae and Droseraceae families, has been reported to possess anticancer activity [2–5]. Besides anticancer effects, plumbagin also exhibited radiosensitizing properties in experimental mouse tumors as well as in tumor cells in vitro [6–8]. However, its cellular mechanism and extent of radiosensitization has not been studied and thus formed the basis of this study. This study shows that a combination of plumbagin and radiation augmented cell growth inhibition when compared to a higher radiation dose, thus indicating that plumbagin may be a potential radiosensitizer acting through the induction of apoptosis.

MATERIALS AND METHODS

Chemicals

Plumbagin was purchased from Sigma (St. Louis, MO). Stock solution of plumbagin was made in dimethyl sulphoxide (DMSO) (Sigma). The final concentration of DMSO for all treatments was less than 0.1%. The antibody against Survivin was purchased from Santa Cruz Biotechnology (Santa

Abbreviation: HPV, human papilloma virus.

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Cruz Biotechnology, Inc., CA); those against Bcl-2 and Bax were obtained from Imgenex (San Diego, CA) and those against caspase 3, 8 and 9 from Cell Signalling, Inc. (Beverly, MA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), custom synthesized primers for RT-PCR and TRI reagent for RNA isolation were purchased from Sigma. MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide), was obtained from Himedia (Mumbai, India), ethidium bromide and acridine orange were purchased from Bangalore Genei (Bangalore, India) while TUNEL kits and RT-PCR reagents were purchased from Promega (Madison, WI).

Cell Lines

Human cervical cancer cell lines, HeLa (HPV18 positive), SiHa (HPV16 positive), C33A (HPV negative) and breast adenocarcinoma cells, MCF 7 and normal breast epithelial cells, HBL 100 were obtained from National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in DMEM supplemented with 10% FBS.

Treatment

Cells were irradiated with gamma rays from a Theratron 780C machine at a dose rate of 266 cGy/min. Cells were treated with different doses of radiation (2, 4, 6, 8 and 10 Gy) and with different concentrations (750 nM, 1 μM, 2.5 μM) of plumbagin. To study the combined effect of plumbagin and radiation we did pretreatment with plumbagin before irradiation, co-treatment of plumbagin and radiation and also replenishment of plumbagin after irradiation. Co-treatment of plumbagin with radiation was found to be most effective in inducing maximum cell death. Different combinations of plumbagin and radiation were tried and the treatments finalized were 750 nM plumbagin, 2, 10 Gy and a combination of 750 nM plumbagin with 2 Gy radiation, for all the experiments. For combined treatment, cells were treated with plumbagin 1 h before irradiation. The MTT assay was done 48 h after the respective treatments and all the other experiments were done 12 h after treatment.

Cytotoxicity Assay

Cytotoxic effect of plumbagin on cervical cancer cells was detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay as described earlier [9]. To assess whether the cytotoxicity of plumbagin is specific for cancer cells, we compared the cytotoxicity of plumbagin on human breast adenocarcinoma cells, MCF 7 and human breast epithelial cells, HBL 100. The experiments were performed in triplicate. Cell survival was expressed as percentage over the untreated control.

Acridine Orange–Ethidium Bromide Staining for Morphological Evaluation of Apoptosis

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy, with an acridine orange and ethidium bromide staining method as described earlier [9]. Acridine orange/ethidium bromide staining uses combination of two dyes to visualize cells with aberrant chromatin organization. Acridine orange was used to visualize the number of cells which has undergone apoptosis, but it cannot distinguish viable from non-viable cells. To achieve this, a mixture of acridine orange and ethidium bromide was used. The differential uptake of these two dyes allows the identification of viable and non-viable cells.

Terminal Deoxynucleotidyl Transferase-Mediated Biotin dUTP Nick End Labeling Assay (TUNEL)

To further confirm apoptotic cells, in situ end labeling of the 3′OH end of the DNA fragments generated by apoptosis-associated endonucleases was performed by using the Dead End apoptosis detection kit (Promega, Madison, WI), according to the manufacturer's protocol.

Loss of Mitochondrial Membrane Potential

This was primarily done according to the protocol supplied by the manufacturers of ApoAlert™ Mitochondrial membrane sensor kit (BD Biosciences Clontech, PaloAlto, CA). MitoSensor aggregates in the mitochondria of healthy cells and fluoresces red. In apoptotic cells the mitochondrial potential is altered and MitoSensor cannot accumulate in mitochondria and remain in the cytoplasm and fluoresces green.

Caspase Activation

The enzymatic activity of caspase 3, caspase 6, caspase 8 and caspase 9 induced by the plumbagin and/or radiation was assayed spectrofluorimetrically. The experiments were performed in triplicate. Briefly, cells were cultured in 90 mm culture plates (2.5×10^6 cells/plate). The cells were treated with the indicated concentrations of the drug/dose of radiation and incubated for 12 h. The cells were scraped for whole cell lysate preparation. Fifty micrograms of total protein was incubated with 50 μM of caspase 3, caspase 6, caspase 8 or caspase 9, flourimetric substrate in a total volume of 500 μl of reaction buffer at 37°C for 1 h in the dark. The released AMC was quantitated using spectrofluorimeter (LS-50B model, Perkin Elmer, MA) with excitation and emission wavelengths 400 and 505 nm, respectively.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) for *Bcl-2*, *Bax*, *Survivin* and *β-2-microglobulin*

Cells were cultured in 90 mm culture plates (2.5×10^6 cells/plate) and treated with or without

different concentrations of plumbagin and/or different doses of radiation for 12 h. Total RNA was isolated from them using TRI reagent according to the manufacturer's recommendations. Five micrograms of total RNA extracted were subjected to cDNA synthesis at 37°C in a 25 μl reaction mix containing 200 U of MMLV Reverse transcriptase in 1× reaction buffer with 2 μg of random hexamer; 6 U of RNAasein; 100 μM dNTP mix for 1 h. The enzyme was inactivated at 90°C for 4 min and quick chilled.

Three microliters of cDNA was used for RT-PCR for genes *Bcl-2*, *Bax*, *Survivin* and *β-2-microglobulin*. Each PCR cycle consisted of an initial denaturation at 94°C for 3 min, followed by melting at 94°C for 30 s, annealing (Table 1) for 40 s and extension at 72°C for 90 s and at the end of 35 cycles a final extension at 72°C for 3 min for *Bcl-2*, *Bax* and *Survivin*. The PCR cycle for *β-2-microglobulin* consisted of an initial denaturation at 95 for 2 min, followed by melting at 94°C for 1 min, annealing (Table 1) for 30 s and extension at 72°C for 30 s and at the end of 38 cycles a final extension at 72°C for 2 min. Expression values for each PCR product were normalized to their respective *β-2-microglobulin* expression.

Western Blot Analysis for Bcl-2, Bax, Survivin, Caspases 3, 8 and 9

Cells were cultured in 90 mm culture plates (2.5×10^6 cells/plate) and treated with or without different concentrations of plumbagin and/or different doses of radiation for 12 h. Western blotting was done using 50 μg of total protein for the detection of protein levels of Bcl-2, Bax, Survivin, Caspases 3, 8 and 9. Total protein was extracted from monolayer cells treated with and without plumbagin and/or radiation as described earlier [9]. Densitometric analysis of the bands was done and normalized with β-actin.

Statistical Analysis

All statistical calculations were carried out with the Statistical Package for Social Sciences (SPSS) software program. Values are expressed as the mean ± SE. The differences among the mean values from at least three independent experiments were analyzed with one-way ANOVA followed by Tukey post hoc *t*-test analysis. Differences between means were considered statistically significant at $P < 0.05$.

RESULTS

Plumbagin in Combination With Radiation Augmented Cell Growth Inhibition in Cervical Cancer Cells

Plumbagin induced cytotoxicity in HeLa, SiHa and C33A cells in a time and concentration-dependent manner (Figure 1A). C33A cells were found to be more sensitive to radiation and plumbagin. A combination of 2 Gy radiation with 750 nM plumbagin augmented the cell growth inhibition more effectively than a radiation dose of 10Gy. SiHa and C33A cells were sensitive to a combination of 750 nM plumbagin and 2 Gy of radiation, cell viability was markedly lower than on treatment with 10 Gy of radiation ($P < 0.001$) (Figure 1B). The radiosensitizing effect of a combination of 2 Gy radiation and 750 nM plumbagin on the cells is a synergistic effect. This was confirmed by calculating the fractional inhibitory concentration (FIC) [10]. Cell viability in HeLa cells after treatment with 10 Gy and a combination of 750 nM plumbagin and 2 Gy irradiation was almost the same (Figure 1B). Our results suggest that the radiosensitizing action of plumbagin was most effective in the C33A cells.

A comparative analysis of the cytotoxicity of plumbagin on human breast epithelial cells, HBL 100 and human breast adenocarcinoma cells, MCF 7; showed that cell death induced by 5 μM plumbagin in the cancer cells was almost threefold higher than in the HBL-100 cells.

Plumbagin and/or Radiation Induce Apoptosis Most Effectively in C33A Cells

To confirm whether the cytotoxic effects induced by plumbagin in these cells involves apoptotic changes, cervical cancer cells were treated with plumbagin and/or radiation for 12 h and stained with acridine orange–ethidium bromide. The number of apoptotic cells was highest when treated with a combination of 750 nM plumbagin and 2 Gy radiation in all the three cell lines (Figure 2). TUNEL positive nuclei were also highest when treated with a combination of 750 nM plumbagin and 2 Gy

Table 1. Primer Sequence

Target gene	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)
Bcl-2	F: 5′GCTTTTCCTCTGGGAAGGAT3′; R: 5′ATCCCACTCGTAGCCCCTCT3′	111	63
Bax	F: 5′TGCCTCAGGATGCGTCCACC3′; R: 5′ACTCGGAAAAAGACCTCTCGG3′	136	60
Survivin	F: 5′ATGGGTGCCCCGACGTTGCC3′; R: 5′AAGGAAAGCGCAACCGGACG3′	262	59
β-2-Microglobulin	F: 5′AAGAGAGGCATCCTCACCCT3′; R: 5′TACATGGCTGGGGTGTTGAA3′	124	50

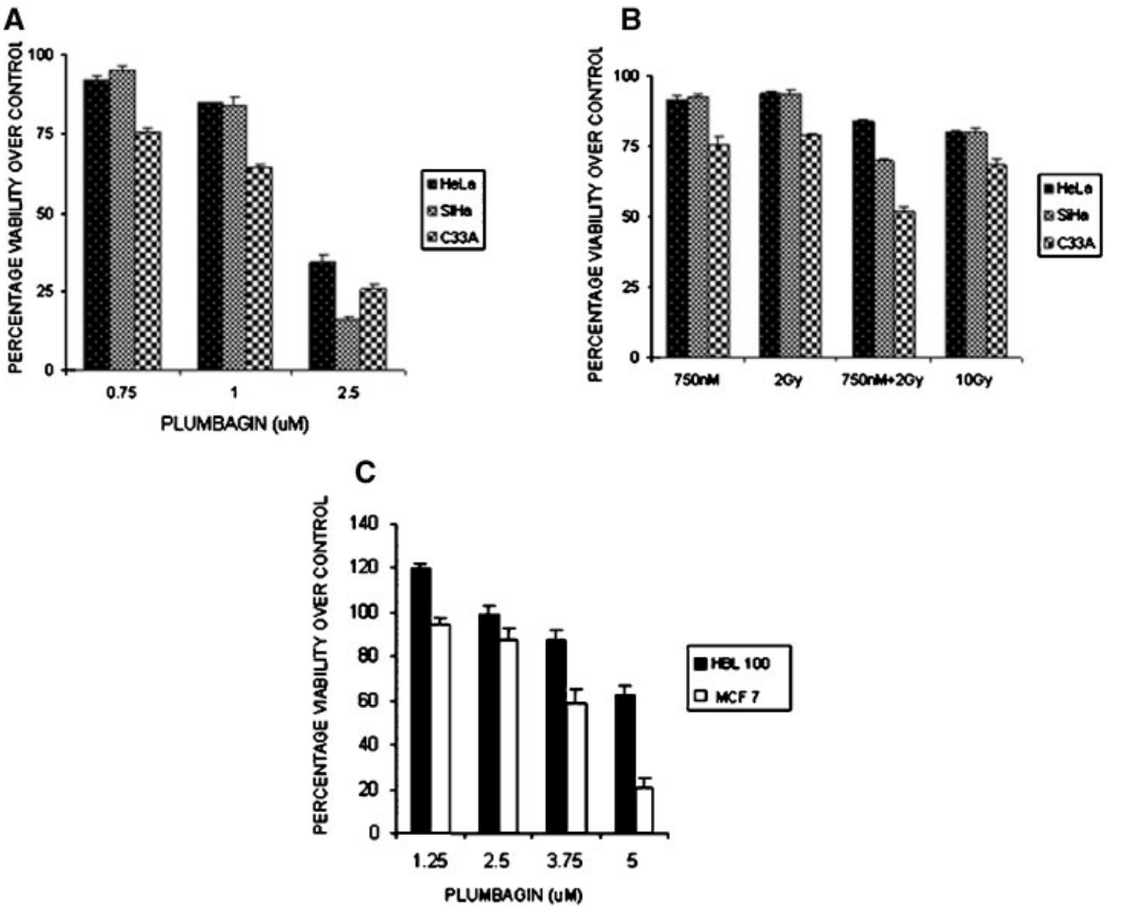

Figure 1. Analysis of cell viability of cervical cancer cells (HeLa, SiHa, C33A) after treatment with (A) plumbagin, (B) plumbagin and/or radiation. (C) Analysis of cell viability of human breast epithelial cells HBL 100 and human breast adenocarcinoma cells MCF 7 after treatment with plumbagin. Cells grown in 96-well plates were then treated with or without the indicated doses for 48 h. Cell viability was assessed by MTT assay as described in Materials and Methods. All results are expressed as the mean percentage of control ± SD of quadruplicate determinations from three independent experiments. The differences among the mean values were analyzed with one-way ANOVA followed by Tukey post hoc *t*-test analysis.

radiation in all the three cell lines (Figure 3). Similar results were obtained while assessing the loss of mitochondrial membrane potential (Figure 4).

Activation of Effector Caspases Confirms That the Mode of Action of Irradiation and Plumbagin Involves Caspase-Dependent Pathways of Apoptosis

Caspases are a family of cysteine proteases that are activated during the execution phase of the apoptotic process. Activation of caspases 3, 6, 8 and 9 was assessed by the spectrofluorimetric caspase activation assay. Activation of all the caspases analyzed was greater on combination treatment when compared to a radiation dose of 10 Gy in both C33A and SiHa cells ($P < 0.001$). Although HeLa cells showed an insignificant increase in activation of caspases 6, 8 and 9, activation of caspase 3 was lower on combination treatment than 10 Gy. The combination dose showed a fivefold increase in the activation of caspase 3 in C33A cells ($P < 0.001$) (Figure 5C).

Activation of caspases in C33A cells was confirmed by Western blotting. The cleaved active bands of caspases 3, 8 and 9 were observed, after all the four treatments. The cleaved active bands of caspase 8 and caspase 3 were observed as very intense bands in the samples treated with the combination dose and 10 Gy (Figure 6). However, there exists considerable heterogeneity among the expression levels of the different caspases induced by plumbagin and/or radiation, this could be because of the activation of specific caspases in a cell specific hierarchical manner.

Plumbagin and/or Radiation Decreases Expression of Survivin

Survivin mRNA expression was decreased in HeLa and C33A cells after treatment with a combination of 750 nM plumbagin and 2 Gy radiation, when compared to the expression after treatment with 10 Gy (Figure 7). *Survivin* mRNA levels in SiHa cells

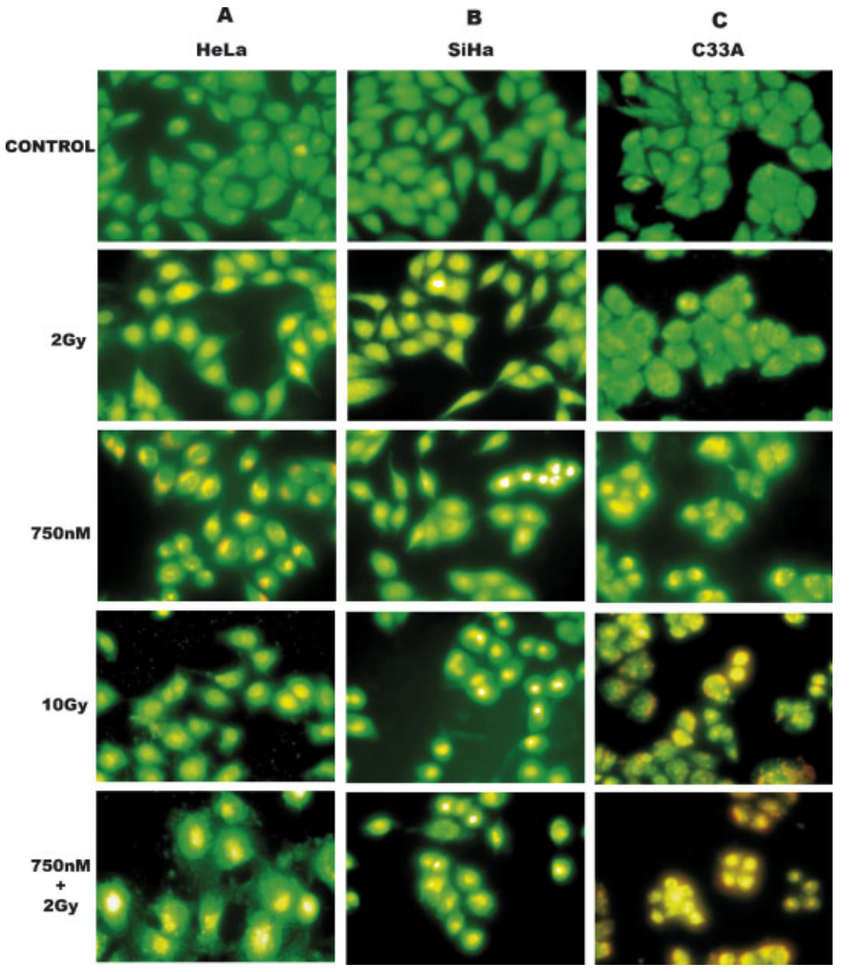

Figure 2. Changes in nuclear morphology induced by plumbagin and/or radiation. (A) HeLa, (B) SiHa and (C) C33A cells were seeded on 30 mm dishes and then treated as shown in the figure for 12 h. After washing with phosphate buffered saline, the cells were stained with a mixture of acridine orange–ethidium bromide solutions. Cells were viewed under fluorescent microscope and photographed (magnification 400×) as described under Materials and Methods. The experiment was repeated twice with similar results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

did not show any remarkable change after the different treatments.

At the protein level, the expression of *Survivin* in C33A cells was much greater than in HeLa and SiHa cells. Survivin protein levels, in all the three cell lines, were decreased as the intensity of the treatment increased (Figure 8A). However, it was observed that treatment with plumbagin and/or radiation did not bring about any pronounced change in the expression levels of *survivin* protein when the values after quantitation were taken into consideration (Figure 8B).

Plumbagin and/or Radiation Regulates Expression of Bcl-2 and Bax

Bax is a proapoptotic member of the *Bcl-2* family of proteins. To check whether plumbagin regulates the expression of *Bax* in inducing apoptosis, we looked at the mRNA level expression by RT-PCR. We observed that the expression of *Bax* was the highest when treated with a combination of 750 nM plumbagin and 2 Gy radiation in SiHa and HeLa cells, but in C33A cells Bax expression was highest in the samples treated with 10 Gy radiation (Figure 7).

The protein level expression of Bax was found to be the highest on treatment with a combination of 750 nM plumbagin and 2 Gy radiation in HeLa cells, hence confirming the RT-PCR results. Bax protein expression in C33A cells was the highest on treatment with 10 Gy radiation (Figure 8). *Bcl-2* mRNA and protein expression in HeLa cells was highest on treatment with a combination of 750 nM plumbagin and 2 Gy of radiation (Figures 7 and 8) and this correlates with our MTT assay results.

DISCUSSION

Radiotherapy is a mode of cancer treatment known to induce cell death in tumors. Radiotherapy

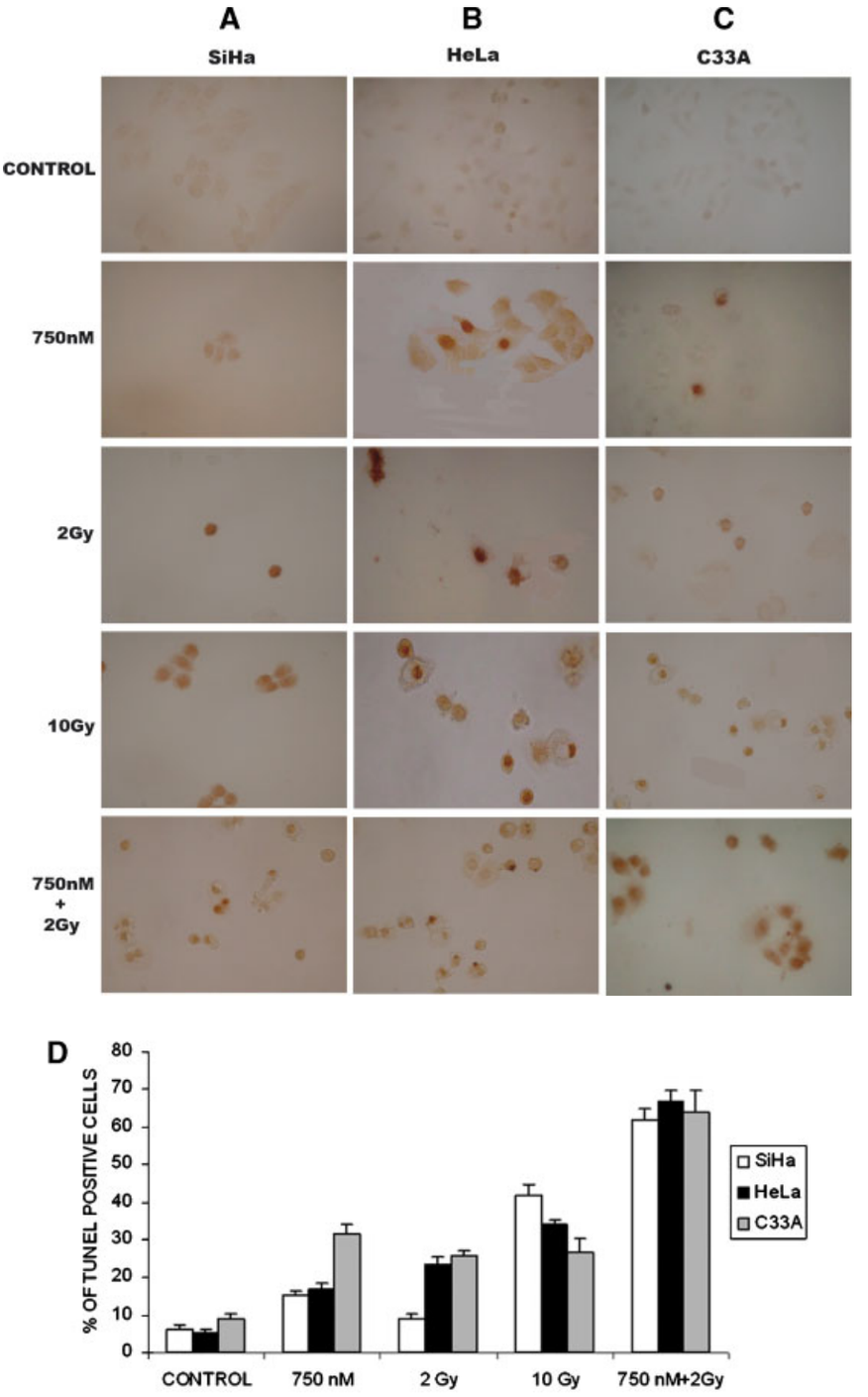

Figure 3. DNA fragmentation induced by plumbagin and/or radiation as determined by TUNEL assay. (A) SiHa, (B) HeLa and (C) C33A were grown on cover slips and treated as indicated for 12 h. The cells were fixed, permeabilized with 0.2% Triton X-100, end labeled with Terminal deoxynucleotidyl transferase reaction mix and the TUNEL reactivity was visualized (magnification 400×) as described in Materials and Methods. (D) Quantitation of apoptotic nuclei was done by counting the cells in random fields and represented as bar diagram. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

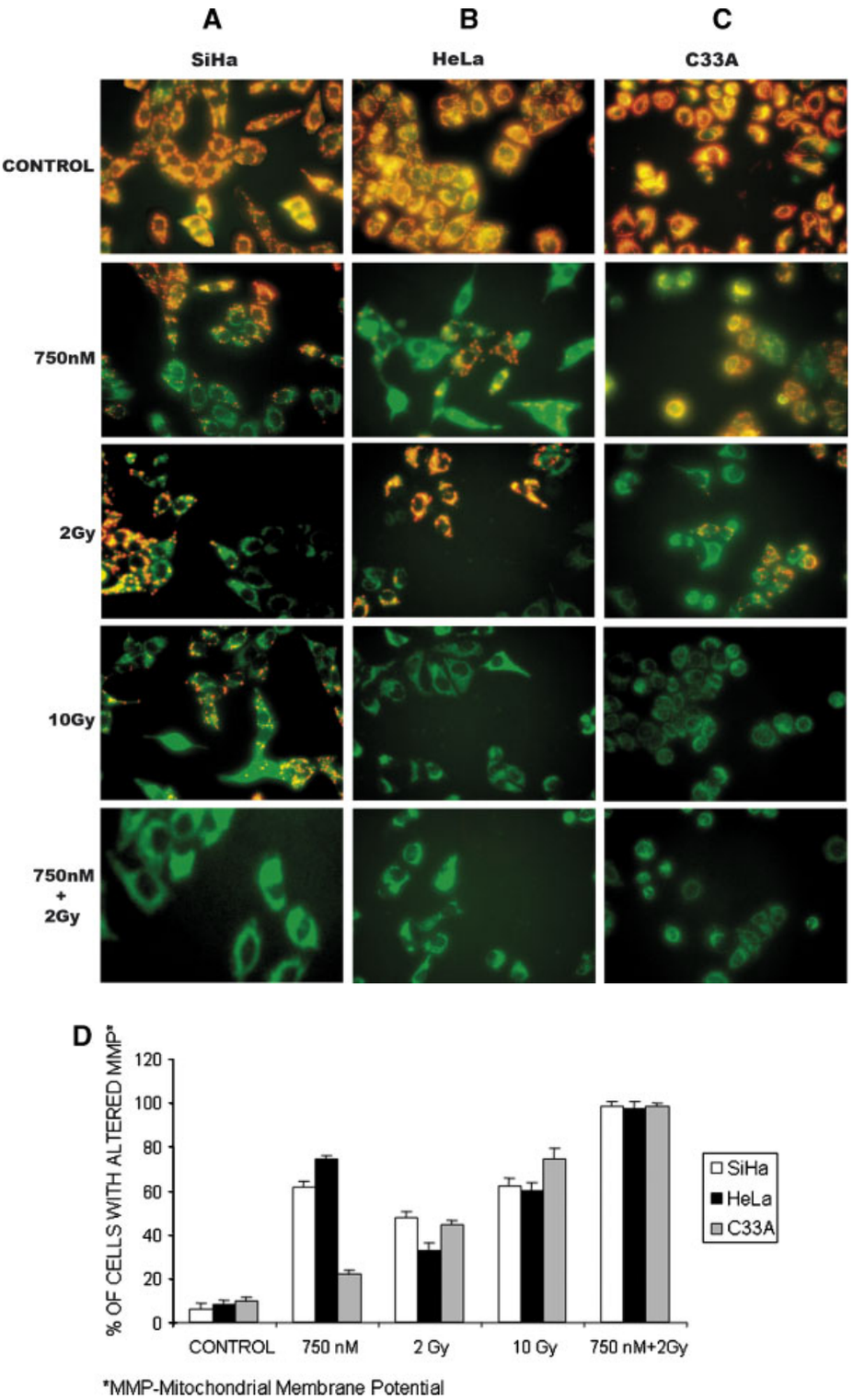

Figure 4. Loss of mitochondrial membrane potential ($\triangle\Psi_m$). (A) SiHa, (B) HeLa and (C) C33A. The cells after treatment were stained with a cationic dye, which aggregates in the mitochondria of healthy cells and fluoresces red. In apoptotic cells the mitochondrial potential is altered and MitoSensor cannot accumulate in mitochondria and remain in the cytoplasm and fluoresces green (magnification 400×). (D) Quantitation of cells with altered mitochondrial membrane potential was done by counting the cells in random fields and represented as bar diagram. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

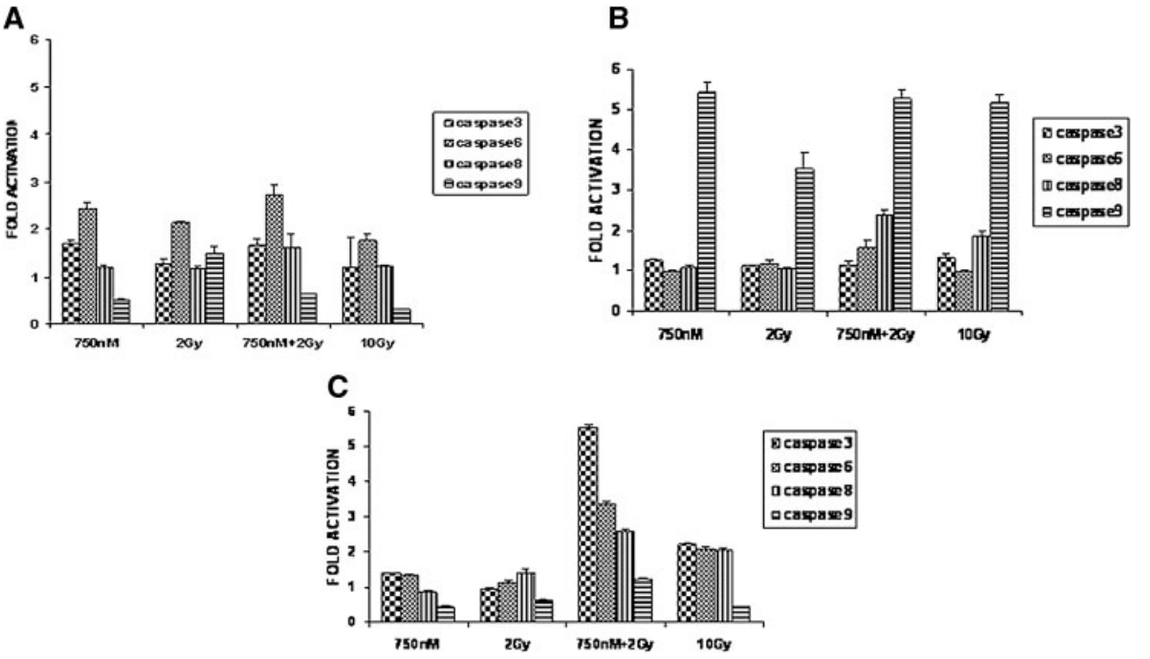

Figure 5. Activation of caspase 3, 6, 8 and 9 by plumbagin and/or radiation in (A) SiHa, (B) HeLa and (C) C33Acells. Cervical cancer cells were treated as indicated for 12 h. Cells were lysed and centrifuged and the supernatant was used for assaying caspase activity, with substrates for caspase 9 (Ac-LEHD-AFC), caspase 8 (Ac-IETD-AFC), caspase 6 (Ac-VEID-AFC) and caspase 3 (Ac-DEVD-AFC) in a reaction buffer at 37°C for 1 h and caspase activation as described under Materials and Methods. The experiment was repeated another time with similar results and the caspase activity was expressed as fold activation over the untreated control.

is limited by the total dose that can be given without damaging normal tissue. Plumbagin has been reported to exhibit radiosensitizing properties in experimental mice [6]. However the underlying mechanism has not been addressed. Plumbagin has been reported to possess anticancer activity and to induce apoptosis in cervical cancer cells by reactive oxygen species (ROS) generation [11]. Hence we hypothesized that plumbagin could also have properties that modify the effects of radiation and treatment of cervical cancer cells with radiation alone and in combination with plumbagin may help in augmenting the anticancer effect of plumbagin.

The effects of plumbagin and/or radiation on three different cervical cancer cell lines were analyzed.

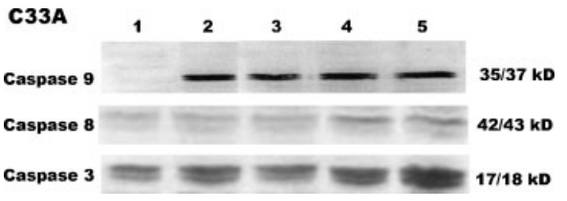

Figure 6. Activation of caspases 3, 8 and 9 by Western blotting. Lane 1: Control, Lane 2: 2 Gy, Lane 3: 750 nM plumbagin, Lane 4: 750 nM plumbagin + 2 Gy, Lane 5: 10 Gy. C33A cells were plated in 90 mm dishes and treated as mentioned in Materials and Methods. Cells were then harvested and whole cell lysates prepared. Fifty micrograms of the cell lysate was subjected to SDS-PAGE and the caspases were detected using specific antibodies.

Plumbagin and radiation induced cell death in all the three cell lines in a time and concentration-dependent manner. Cell viability was assayed by the reduction of MTT at 24 and 48 h after treatment. The radiosensitizing action of plumbagin was most pronounced 48 h after treatment (Figure 1). Co-treatment of plumbagin with radiation was found to be the most effective in inducing cell death. A combination of 2 Gy radiation with 750 nM plumbagin augmented the cell growth inhibition more effectively than a radiation dose of 10 Gy (Figure 1B). SiHa and C33A cells were very sensitive to the combination treatment when compared to a radiation dose of 10 Gy, but HeLa cells were resistant to both treatments. The radiosensitizing effect of a combination of 2 Gy radiation and 750 nM plumbagin on the cells could be because of a synergistic effect. Previous studies have demonstrated sensitivity to radiation to be enhanced in aspirin pretreated HeLa TG cells and that aspirin had an additive role for amplifying the radiotherapeutic effect in cervical cancer cells [12]. A recent study on cellular response to chemotherapy and radiation in different cervical cancer cell lines also showed that HPV negative C33A was more sensitive to radiation [13]. Our results also suggest that the radiosensitizing action of plumbagin was most effective in the HPV negative C33A cells. But as of now there is no evidence to suggest the combined effect of plumbagin with radiation is

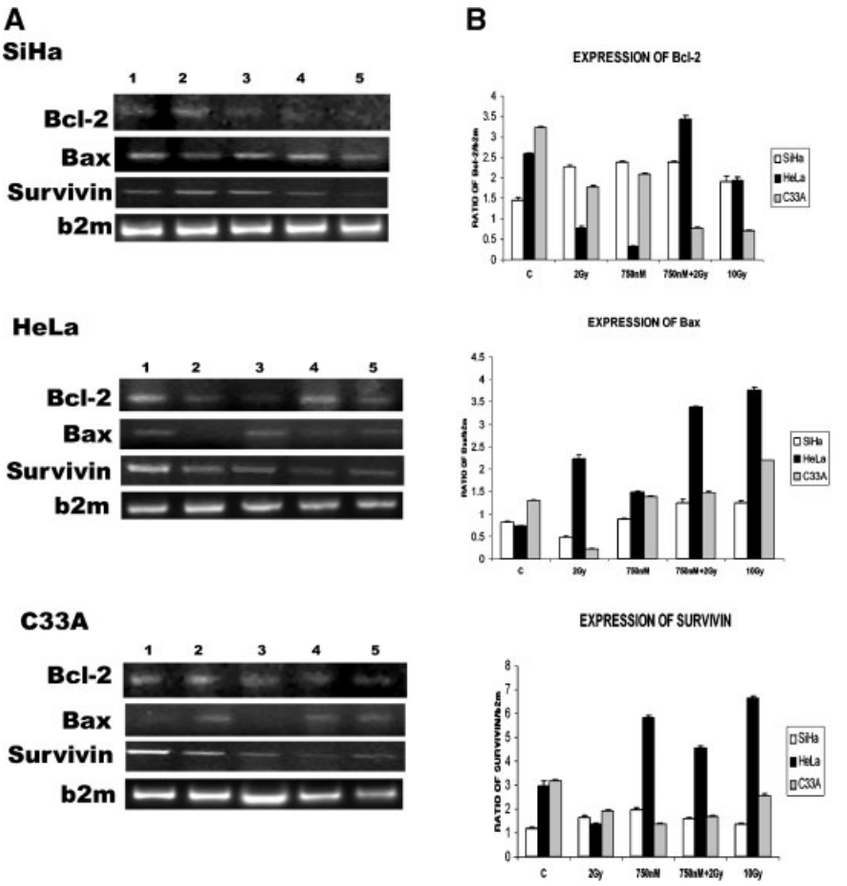

Figure 7. (A) RT-PCR analysis of mRNA expression of *Bcl-2*, *Bax*, *Survivin* and *β-2-microglobulin* in HeLa, SiHa and C33A cells. Lane 1: Control, Lane 2: 2 Gy, Lane 3: 750 nM plumbagin, Lane 4 750 nM plumbagin + 2 Gy, Lane 5: 10 Gy. (B) Bands of interest were further analyzed by densitometry. Total RNA was extracted from the cells treated with plumbagin and/or radiation for 12 h. Five micrograms of RNA was used for RT-PCR. Expression values for each PCR product were normalized to their respective β-2-microglobulin expression.

modulated by the presence of HPV. The cancer specificity of Plumbagin was also analyzed by treating human breast epithelial cells, HBL 100 and human breast adenocarcinoma cells, MCF 7, with different doses of plumbagin. We could confirm that plumbagin induced cytotoxicity in MCF 7 cells was almost 3 times greater than in HBL 100 cells.

Plumbagin has been shown to induce apoptosis in ME180 cervical cancer cells and BG-1 ovarian cancer cells [11,14]. To explore the mechanism of cell death in cervical cancer cells by plumbagin and radiation we studied the induction of apoptosis. Results show that cell growth inhibition in HeLa, SiHa and C33A cells, by plumbagin, radiation and a combination of the two is by apoptosis and this was confirmed by the loss of mitochondrial membrane potential (Figure 4) and TUNEL assay (Figure 3).

Caspases are a family of cysteine proteases that are activated during the execution phase of the apoptotic process [15,16]. Once activated, caspases cleave and activate downstream caspases [16]. A combination dose of 750 nM plumbagin and 2 Gy radiation showed a fivefold increase in the activation of caspase 3 in C33A cells (Figure 5C). Activation of caspase 6, 8 and 9 was also greater on combination treatment when compared to a radiation dose of 10 Gy. In SiHa cells the activation of caspases was not as marked as in C33A cells, but the activation of caspases 3, 6, 8 and 9 was greater on treatment with a combined dose than when treated with 10 Gy (Figure 5A). Combination treatment in HeLa cells showed a greater activation of caspase 6 and 8 than when treated with 10 Gy, whereas there was no remarkable change in the levels of caspase 9 on all treatments (Figure 5B). Plumbagin mediated activation of caspase 3 and caspase 9 has been shown earlier in ME180 cervical cancer cells [11]. Recent studies using Shikonin and Ukrain have shown that HeLa cell apoptosis is induced through the activation of caspases [17,18]. K562 cells treated with radiation and herbimycin A had an accelerated cell death and induced a *p53* independent apoptosis. This apoptotic

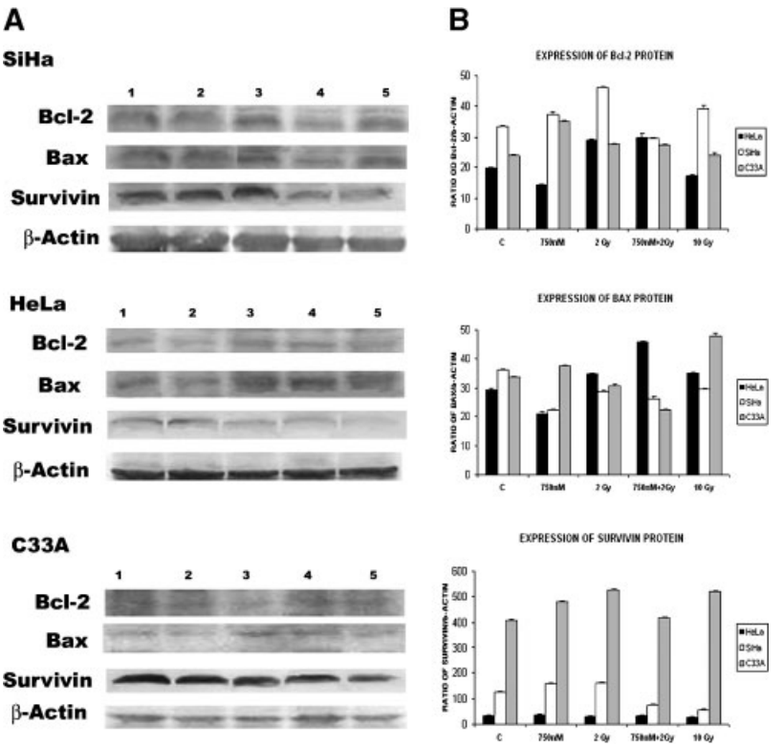

Figure 8. (A) Expression of Bcl-2, Bax and Survivin by Western blotting. Lane 1: Control, Lane 2: 2 Gy, Lane 3: 750 nM plumbagin, Lane 4: 750 nM plumbagin +2 Gy, Lane 5: 10 Gy. (B) Bands of interest were further analyzed by densitometry. Cells were plated in 90 mm dishes and treated as mentioned in Materials and Methods. Cells were then harvested and whole cell lysates prepared. Fifty micrograms of the cell lysate was subjected to SDS-PAGE and the proteins were detected using specific antibodies. The same blot was reprobed with β-actin antibody. Bands were analyzed by densitometry and normalized to β-actin expression.

pathway was dependent upon an initial hyperpolarization of the mitochondrial inner membrane, following the release of cytochrome *c* and subsequent caspase 3 activation [19]. Our results also show the involvement of the caspase-dependent pathways of apoptosis. There exists considerable heterogeneity among the expression levels of the different caspases induced by plumbagin and/or radiation (Figure 6), this could be because of the activation of specific caspases in a cell specific hierarchical manner.

Apart from the activation of caspases we also studied the expression of survivin. *Survivin* has recently been identified as a novel member of the inhibitor of apoptosis (IAP) gene family. The product of this gene not only suppresses apoptosis but also controls cell division. *Survivin* is undetectable in most terminally differentiated normal tissues but is expressed in embryonic and fetal organs and is present in most malignant tumors [20]. *Survivin* mRNA expression was decreased in HeLa and C33A cells after treatment with a combination of 750 nM plumbagin and 2 Gy radiation, when compared to the expression after treatment with 10 Gy (Figure 7). Plumbagin has been shown to downregulate the expression of *survivin* [21]. Plumbagin and radiation treatment reduced the expression of *survivin* in all the three cell lines, showing an increased susceptibility for apoptosis to occur. Therefore induction of apoptosis and inhibition of IAPs may be a mechanism of action of plumbagin with radiation.

Human papilloma viruses are thought to play an important role in the development of cervical cancer [22]. By interfering in the cell cycle, the viral oncoproteins (E6 and E7) can induce the immortalization of the host cell. Experiments performed in different cancer cell lines and with different E6 mutants indicated that the effect of E6 on the survivin promoter is largely dependent on p53 status. As E6 is able to interact with p53 and induces its ubiquitin-dependent degradation, it appears that the transactivation effect of E6 on survivin is mediated by the p53 degradation pathway [20]. Studies on sarcoma cells suggest that the wt-p53-caspase pathway is of importance for the radiosensitization induced by targeting survivin, which may have an impact on future gene therapeutical treatments [23].

Members of the gene family that includes *Bcl-2* and *Bax* are involved in the control of apoptosis in a range of different cell types. The ability of *Bcl-2* to inhibit

apoptosis is dependent on expression of *Bcl-2* and on the formation of hetero- and homodimers between members of the *Bcl-2* family [24]. On the other hand, the *BAX* gene is an apoptosis-promoting member of the *BCL-2* gene family, and apoptosis is known to be accelerated when the *BAX* function is predominant [25]. Our results show marginal changes in the expression levels of *Bcl-2* and *Bax* in all the three cell lines (Figures 7 and 8). It has been observed that unchanged expression of the pro-apoptotic protein Bax and suppression of the anti-apoptotic molecules Bcl-2 and Bcl-x (L) argues for the engagement of the mitochondrial apoptotic pathway [26].

To summarize, our results show that a combination of plumbagin and radiation augmented cell growth inhibition when compared to a higher radiation dose, thus indicating that plumbagin may be a potential radiosensitizer. C33A cells were found to be the most sensitive to the combination treatment. This is the first report confirming the radiosensitizing action of plumbagin in cervical cancer cells. Plumbagin induces apoptosis in all the three cervical cancer cells via the caspase-dependent pathways. The documented effect may also be because of the inhibition of survivin levels. Our study shows that although cell growth inhibition is highest in combination treatment, mechanism of action is different in each cell line owing to their molecular alterations. The results from the three cell lines varied with respect to the expression of various proteins in response to plumbagin, radiation and its combination. Therefore, in vivo tumors (more heterogenic) may behave much differently and a lot more studies have to be done before any work could be planned in an animal model of cervical cancer. Nevertheless this study shows the potential of plumbagin in augmenting radiation effects.

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