



# Selectively enhanced radiation sensitivity in prostate cancer cells associated with proteasome inhibition

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**Abstract.** The purpose of this study is to evaluate the utility of MG-132, a broad spectrum proteasome inhibitor, to selectively enhance radiation sensitivity in prostate cancer without affecting normal surrounding urothelial tissue. PC3 prostate cancer cells and normal URO-tsa bladder epithelial cells were treated with or without MG-132 and exposed to 0, 2, 4, or 6 Gy radiation. Cell viability and clonogenic survival assays were performed, and nuclear factor kappa-B (NF- $\kappa$ B) activity was evaluated with electrophoretic mobility shift assay (EMSA). MG-132 was associated with decreased cell viability (between 24% and 33%) and clonogenic survival (between 71% and 88%) alone and in combination with radiation in PC3 cells. MG-132 had no effect on cell viability or clonogenic survival following radiation in URO-tsa cells. Constitutive and radiation-induced NF- $\kappa$ B binding activity was higher in PC3 cells compared with URO-tsa cells. Furthermore, MG-132 at concentrations associated with reductions in cell viability and clonogenic survival inhibited NF- $\kappa$ B binding activity in PC3 cells with no effect in URO-tsa cells. These results provide strong evidence that proteasome inhibition and concomitant NF- $\kappa$ B inhibition can be used to selectively enhance tumor radiation sensitivity in prostate cancer without affecting normal surrounding bladder tissue.

## Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer death afflicting men in the United States (1). Radiotherapy in the form of external beam radiation and interstitial brachytherapy are well established and commonly employed as curative therapy for locally confined prostate cancer. Radiation is also commonly used

for salvage therapy in individuals who have failed radical prostatectomy; however, radiation therapy for primary or salvage treatment can be associated with impotence, urethral stricture, radiation cystitis, and radiation proctitis. Improved response to therapy and improved quality of life by reducing morbidity are current areas of interest in prostate cancer research.

Proteasomes are multi-subunit cellular structures responsible for the non-lysosomal degradation of most intracellular proteins and molecules including proteins involved in transcriptional regulation, stress response, survival, and response to external stimuli such as inflammation and hormonal regulation (2,3,13,14). Tumor suppressor gene and oncogene products known to interact with the proteasome include APC, RB1, RAS, MYC, FOS, ABL and many others demonstrating involvement in a wide variety of tumors and tissues. Proteasome inhibition has been demonstrated to increase differentiation, decrease proliferation, increase sensitivity to chemotherapy, and increase radiation sensitivity in numerous tumors and cancer cell lines (4-7,13). Proteasome inhibition is also known to modulate the activity of nuclear factor kappa-B (NF- $\kappa$ B), a transcription factor associated with regulation of apoptosis, angiogenesis, inflammation, cellular stress response, and tumor survival. The proteasome can affect NF- $\kappa$ B by degrading the inhibitory subunit and allowing active NF- $\kappa$ B to translocate to the nucleus and modulate transcription (8,15,16).

Proteasome inhibition has been demonstrated to increase tumor cell death, enhance chemotherapy, and increase radiation sensitivity in prostate cancer cells (5,9-11). MG-132, a broad spectrum proteasome inhibitor, decreased bombesin-induced angiogenesis in PC3 cells concomitant with NF- $\kappa$ B inhibition implicating proteasomal regulation of NF- $\kappa$ B as a potential mechanism of inhibiting tumor progression (10). Furthermore, proteasomal inhibition with saquinavir, a human immunodeficiency virus-1 proteasome inhibitor, sensitized PC3 cells to radiation at doses associated with NF- $\kappa$ B inhibition (5). These studies support the hypothesis that decreased NF- $\kappa$ B activity through proteasome inhibition could result in decreased tumor growth and radiation survival.

Selective enhancement of radiation sensitivity in tumor cells without affecting surrounding normal tissues could be utilized to reduce morbidity associated with radiation. The purpose of this study was to evaluate the potential utility of proteasome inhibition in modulating NF- $\kappa$ B activity and

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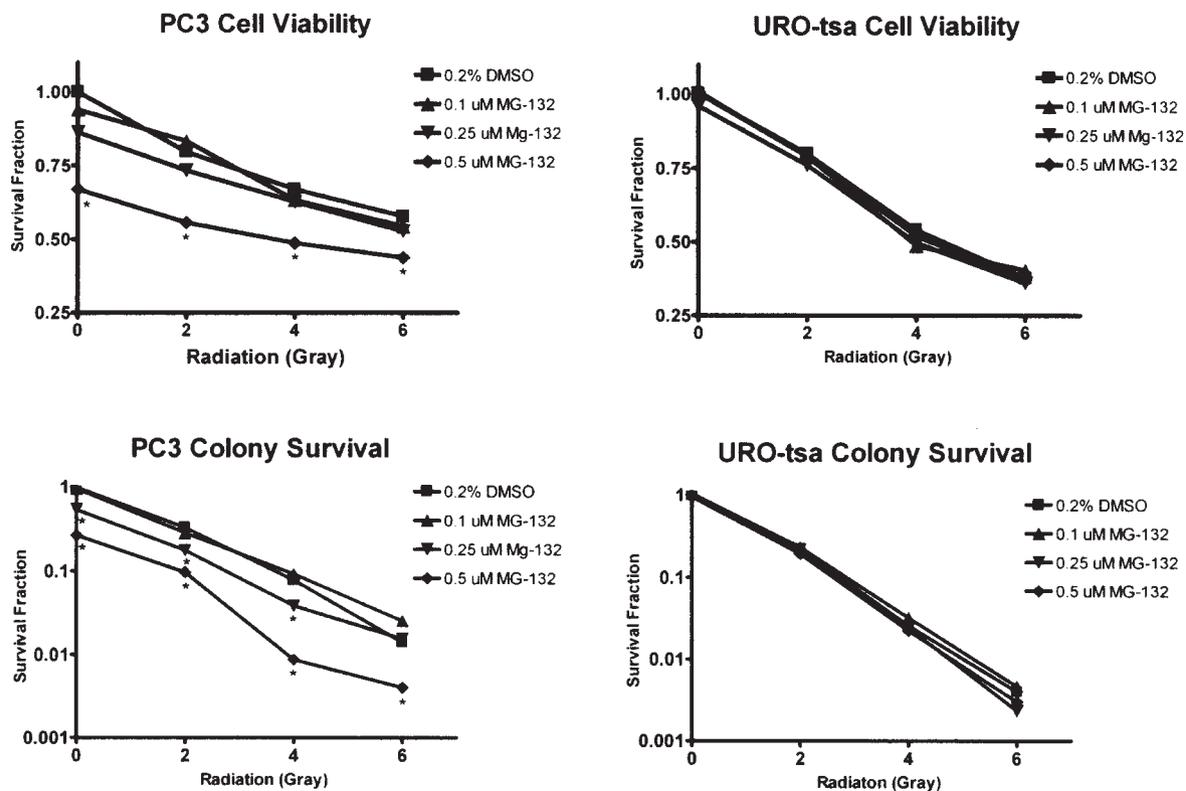


Figure 1. The effects of MG-132 on cell viability and clonogenic survival in PC3 and URO-tsa cells. Cells were treated with MG132 for 2 h prior to irradiation with 0, 2, 4, or 6 Gy and cell viability was measured 72 h following radiation exposure. Colonies for clonogenic survival were counted 10 days after plating for PC3 cells and 12 days after plating for URO-tsa cells. \*Denotes statistically significant difference from DMSO-treated controls in each radiation dose ( $p < 0.05$ ).

selectively enhancing radiation sensitivity in the p53 null PC3 prostate cancer cell line without affecting radiation sensitivity in the non-tumorigenic urothelial cell line URO-tsa (12). If the radiation sensitivity of prostate cancer cells can be selectively enhanced without affecting urothelial cells, this evidence could be used to promote clinically relevant studies to enhance radiation response in prostate cancer without affecting the adjacent normal bladder epithelium, thereby improving the treatment of prostate cancer without sacrificing quality of life as related to urinary function

## Materials and methods

**Materials.** PC3 cells were obtained from ATCC (Rockville, MD), and URO-tsa cells were a kind gift from Dr Nyseo Unimye (Department of Urology, Virginia Commonwealth University). RPMI media was obtained from Invitrogen Gibco (Carlsbad, CA). Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). MG-132 was obtained from Calbiochem (LaJolla, CA). Protein concentration reagent was obtained from Bio-Rad (Hercules, CA). Oligonucleotides for electrophoretic mobility shift assays (EMSA) were obtained from Promega (Madison, WI). Radiation was delivered using a Faxitron RX-650 X-ray source (Wheeling, IL) and operated at 125 kV and 5 mA with 1 mm of aluminum filtration.

**Cell viability assay.** PC3 cells and URO-tsa cells were grown in RPMI media containing 10% FBS and 1% penicillin/streptomycin. T25 flasks were plated with either 200,000

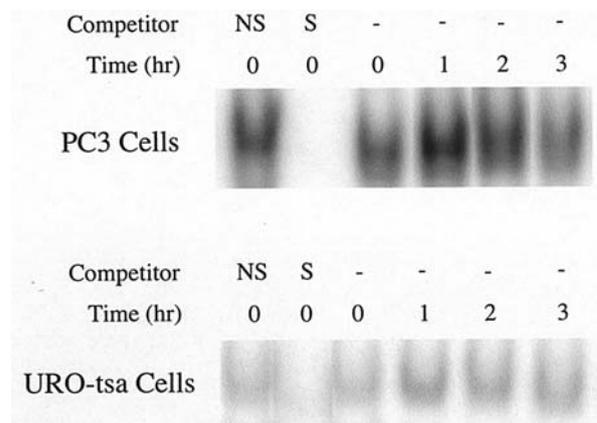


Figure 2. The effect of MG-132 on NF- $\kappa$ B activity in PC3 and URO-tsa cells following 6 Gy irradiation. Cells were treated with 0 (sham) or 6 Gy irradiation and collected 1, 2, and 3 h following irradiation. NF- $\kappa$ B activity was assessed using EMSA. Self (S) and non-self (NS) oligonucleotides were used to compete for radiolabelled NF- $\kappa$ B probe thereby demonstrating specificity of the probe.

PC3 cells or 400,000 URO-tsa cells and allowed to grow for 3 days in a humidified incubator supplemented with 5% CO<sub>2</sub> at a temperature of 37°C. Cells were then treated with MG-132 at concentrations of 0.1, 0.25, or 0.5  $\mu$ M suspended in dimethylsulfoxide (DMSO to a total dose of 0.2% in each flask) or with 0.2% DMSO as a control for 2 h prior to treatment with 0, 2, 4, or 6 Gy radiation. Medium containing DMSO or MG-132 was removed 24 h after irradiation, and fresh medium was introduced. Cells were allowed to grow

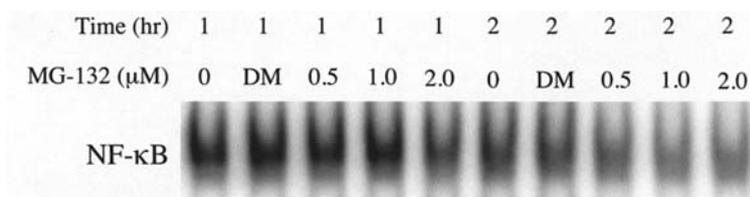


Figure 3. NF- $\kappa$ B activity in irradiated PC3 cells treated with MG-132. Cells were treated with RPMI (0), DMSO (DM), or 0.5, 1.0, or 2.0  $\mu$ M MG-132 at 2 h prior to irradiation with 6 Gy. Cells were collected and EMSA performed to evaluate NF- $\kappa$ B activity at 1 and 2 h following irradiation.

for a total of 72 h following irradiation. Cell viability was determined using Trypan blue staining with a Beckman-Coulter ViCell A cell counter (Beckman-Coulter, Fullerton, CA). Relative survival rates were obtained by comparing viable cell counts compared with DMSO-treated controls without irradiation.

**Clonogenic survival assay.** Cells were maintained as described above and plated on T25 flasks at concentrations of 200, 1600, and 3200 cells per flask. Cells were plated 24 h prior to MG-132 treatment. Treatments with MG-132 and radiation were performed as described above. The cells were assayed for colony forming ability. After 10-12 days incubation in drug-free medium, the cells were fixed and stained with 0.5% crystal violet in methanol. Colonies with >50 cells were counted as clonogenic survivors. Survival fractions were obtained according to standard protocol by comparing cell counts with plating efficiency of untreated controls.

**Electrophoretic mobility shift assay (EMSA).** Confluent PC3 cells or URO-tsa cells were treated with MG-132 and radiation as described above. Cells were harvested by removing medium, scraping cells into a small volume of phosphate-buffered saline (PBS), centrifugation, and another washing with PBS. Cells were then resuspended in buffer containing 10 mM HEPES-KOH, 1.5 mM  $MgCl_2$ , 10 mM M KCl, 0.2 mM PMSF, 0.5 mM dithiothreitol, 5 mM NaF, 1 mM  $Na_3VO_4$ , leupeptin, aprotinin, and pepstatin. Cells were incubated on ice, and lysed with 10% NP-40. Lysates were centrifuged, supernatant removed, and lysates were resuspended in salt solution containing 10 mM HEPES-KOH, 1.5 mM  $MgCl_2$ , 0.42 mM NaCl, 35% glycerol, 0.2 mM PMSF, 0.5 mM dithiothreitol, 0.2 mM EDTA, 5 mM NaF, 1 mM  $Na_3VO_4$ , leupeptin, aprotinin, and pepstatin, and incubated on ice. Cells were centrifuged and the supernatant removed represented nuclear extracts. For EMSAs, nuclear extracts from three separate experiments were combined to provide an accurate representation of NF- $\kappa$ B activity. Protein concentrations of nuclear extracts were obtained according to the method of Bradford (17).

To evaluate NF- $\kappa$ B activity, EMSAs were performed according to methods described by Promega ([www.promega.com](http://www.promega.com)). Briefly, 15  $\mu$ g of protein from each sample was incubated on ice with binding buffer containing 20% glycerol, 5 mM  $MgCl_2$ , 2.5 mM EDTA, 2.5 mM dithiothreitol, poly dI-dC, and  $^{32}P$ -labeled double-stranded consensus oligonucleotide specific for NF- $\kappa$ B. To show specificity, controls without MG-132 or radiation treatment were incubated with 100-fold excess competitors of non-radiolabelled NF- $\kappa$ B consensus (self) and non-self sequences. The sequence for

the NF- $\kappa$ B consensus oligonucleotide are 5'-AGTTGAGGG GACTTCCAGG-3' and 3'-TCAACTCCCCTGAAA GGGTCC-5'. Nuclear extract incubated with radiolabelled oligonucleotides were loaded onto a 4% polyacrylamide gel and run at 120-130 V for 90-120 min.

**Statistical analysis.** Results were compared using SPSS version 10 with analysis of variance between DMSO-treated controls and MG-132 treatments for each radiation dose.

## Results

For all experiments, both PC3 and URO-tsa cells were maintained on RPMI media containing 10% FBS and 1% penicillin/streptomycin. PC3 cells demonstrated an average doubling time of 20 h and URO-tsa cells doubled every 33 h. To evaluate tumorigenicity of PC3 and URO-tsa cells, 4 million cells were injected into the hind limbs of nude mice (n=4). Tumors formed in 4/4 mice injected with PC3 cells and grew to the point of necessitating euthanization according to IACUC protocols. No tumors formed in any of 4 mice injected with URO-tsa cells by 6 months after injection. DMSO treatment had no observable effect on cell viability, plating efficiency, or NF- $\kappa$ B activity in PC3 or URO-tsa cells.

The effects of MG-132 on cell viability following irradiation are shown in Fig. 1. Cell viability decreased in a dose-dependent fashion with increasing radiation doses for both PC3 and URO-tsa cells. Whereas cell viability after 2 Gy radiation in DMSO-treated PC3 cells was 80% in both PC3 and URO-tsa cells, URO-tsa cell viability was 38% after 6 Gy radiation compared with 58% in PC3 cells. PC3 cells demonstrated reduced cell viability with 0.5  $\mu$ M MG-132 treatment for all doses of radiation, as well as in cells without radiation treatment ( $p < 0.05$ , n=3). Reductions in cell viability compared to DMSO-treated controls ranged from 24% at 6 Gy to 33% at 0 Gy. In contrast, the same concentrations of MG-132 had no effect on cell viability alone or following radiation treatment in URO-tsa cells.

Clonogenic survival in PC3 and URO-tsa cells were comparable to findings observed with cell viability (Fig. 1). Survival fractions at 2 Gy were 0.33 in PC3 cells and 0.22 in URO-tsa cells. The 0.5  $\mu$ M MG-132 treatment reduced survival fractions in PC3 cells at all doses of radiation between 71% and 88%. Furthermore, 0.25  $\mu$ M MG-132 reduced survival fractions in PC3 cells at 0, 2, and 4 Gy radiation (between 46% and 51%). MG-132 had no effect on survival fractions in URO-tsa cells at any radiation dose.

The effect of radiation on NF- $\kappa$ B activity evaluated by EMSA is shown in Fig. 2. PC3 cells demonstrated increased NF- $\kappa$ B activity with a peak at 1 h following irradiation with

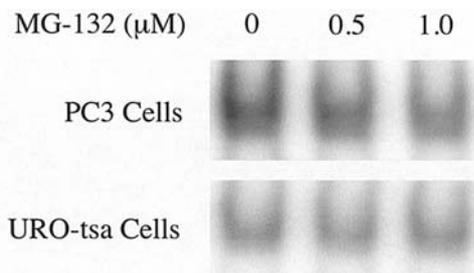


Figure 4. The effect of MG-132 on NF- $\kappa$ B activity in PC3 and URO-tsa cells following irradiation. PC3 and URO-tsa cells were treated with 0, 0.5, or 1.0  $\mu$ M MG-132 2 h prior to irradiation with 6 Gy. Cells were collected and EMSA performed to evaluate NF- $\kappa$ B activity 2 h following 6 Gy irradiation.

6 Gy. A trend toward baseline NF- $\kappa$ B activity was observed at 3 h following irradiation. URO-tsa cells demonstrated a similar pattern with decreased relative NF- $\kappa$ B activity compared with PC3 cells.

To evaluate the effects of MG-132 treatment on NF- $\kappa$ B activity following irradiation with 6 Gy, PC3 cells were treated 2 h prior to 6 Gy irradiation with MG-132 at concentrations ranging from 0 to 2.0  $\mu$ M (Fig. 3). PC3 cells treated with MG-132 demonstrated a decrease in radiation-induced NF- $\kappa$ B activity with 2.0  $\mu$ M MG-132 1 h following irradiation; however, a dose-dependent decrease in NF- $\kappa$ B activity was observed 2 h after irradiation. A decrease in NF- $\kappa$ B activity at 2 h following irradiation was noted with MG-132 concentrations as low as 0.5  $\mu$ M.

To compare the effects of MG-132 treatment on NF- $\kappa$ B activity following irradiation, PC3 and URO-tsa cells were treated with increasing doses of MG-132 (Fig. 4). NF- $\kappa$ B activity 2 h following irradiation was decreased in a dose-dependent manner in PC3 cells. MG-132 had no effect on NF- $\kappa$ B activity in URO-tsa cells. A similar dose-dependent decrease in NF- $\kappa$ B activity was noted in PC3 cells at 3 h following irradiation with no effect on URO-tsa cells (data not shown).

## Discussion

The present studies demonstrate that proteasome inhibition can selectively enhance radiation sensitivity in PC3 cells with no effect on normal URO-tsa cells. Cell viability and clonogenic survival assays clearly demonstrate that MG-132 can additively decrease cell survival following radiation in PC3 cells without affecting URO-tsa cells. Furthermore, MG-132 treatments at the same concentrations associated with enhanced radiation sensitivity produced dose-dependent reductions in NF- $\kappa$ B activity in PC3 cells with no observable changes on NF- $\kappa$ B activity in URO-tsa cells. These results are consistent with the hypothesis that reduced NF- $\kappa$ B activity by proteasomal inhibition is a potential mechanism of selectively enhancing radiation sensitivity in prostate cancer cells.

Constitutive NF- $\kappa$ B was located as an inactive form bound to an inhibitory subunit in the cytoplasm of cells (8,15,16). Inhibitory kappa B (I $\kappa$ B) or an inhibitory NF- $\kappa$ B subunit p100 are constitutively bound to NF- $\kappa$ B rendering the protein inactive in the cytoplasm of cells. Activation of NF- $\kappa$ B by

various stimuli, including inflammation, stress and radiation, involves degradation of the inhibitory subunit and translocation of activated NF- $\kappa$ B to the nucleus to regulate transcription. The proteasome is responsible for the degradation of I $\kappa$ B, as well as proteolytic cleavage of the p100 subunit resulting in activation of NF- $\kappa$ B (2,3,14). Consequently, proteasome inhibition results in decreased degradation of the inhibitory subunits providing a mechanism of preventing translocation of activated NF- $\kappa$ B into the nucleus.

The increase in radiation sensitivity with MG-132 in PC3 cells is consistent with previous studies demonstrating reduced survival in MG-132-treated HD-My-Z Hodgkin cells following radiation administration (18). A 3-h preincubation with 50  $\mu$ M MG-132 decreased survival fractions of HD-My-Z cells following radiation treatment; however, whereas MG-132 was associated with increased apoptosis, there was no effect on constitutive NF- $\kappa$ B activity. Unfortunately, no evidence of the effects of radiation with or without MG-132 treatment was provided in HD-My-Z cells. Furthermore, although specific inhibition of NF- $\kappa$ B binding activity using a dominant negative I $\kappa$ B $\alpha$  construct was associated with complete abolition of NF- $\kappa$ B binding activity, inhibition of NF- $\kappa$ B by dominant negative I $\kappa$ B $\alpha$  did not affect radiosensitivity, suggesting that inactivation of NF- $\kappa$ B by I $\kappa$ B $\alpha$  alone is insufficient for enhanced radiosensitivity.

Proteasome inhibition with 2-3 h pretreatment of 50-60  $\mu$ M saquinavir, a human immunodeficiency virus-1 proteasome inhibitor that specifically inhibits the 26S proteasomal subunit, sensitized PC3 and DU-145 prostate cancer cells to radiation (5). Inhibition of clonogenic survival by saquinavir correlated with inhibition of NF- $\kappa$ B activity following radiation treatment and is consistent with the inhibition demonstrated herein with MG-132. In contrast to the absence of effect in saquinavir-treated cells at 0 Gy with no post-radiation saquinavir exposure, the inhibition of survival fraction in PC3 cells at 0 Gy radiation in this study could be due to the 24 h post-radiation MG-132 exposure.

Studies demonstrate that PC3 cells have a significantly higher constitutive NF- $\kappa$ B activity and proteasome activity than other cell lines evaluated including HD-My-Z Hodgkin cells, LnCaP human prostate cancer cells, SW 1088 astrocytoma cells, ECV 304 bladder carcinoma cells, and A549 non-small cell cancer cells (18). Increased NF- $\kappa$ B activity and concomitant-increased constitutive interleukin-6 expression has also been observed in PC3 and DU-145 prostate cancer cells (19). Compared with URO-tsa cells, PC3 cells further reflect a significant relative increase in constitutive NF- $\kappa$ B activity. The significantly increased constitutive activity in PC3 cells could be associated with several tumor-specific survival advantages that may be responsive to the effects of proteasome inhibition.

No studies to date have evaluated the effects of radiation or proteasome inhibition on URO-tsa cells. Though URO-tsa cells were originally grown in F-12 media containing epidermal growth factor, insulin, and hydrocortisone, URO-tsa cells grew well in RPMI media as described above. To standardize treatments between PC3 and URO-tsa cells, both cell types were grown in identical RPMI media containing FBS and penicillin/streptomycin, thereby eliminating potential confounding variables such as growth factor and steroid



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 lent in radiation response. URO-tsa cells were stably maintained through 50 passages until growth was arrested and cells stopped dividing. Evaluation of tumorigenicity of URO-tsa cells grown in RPMI media demonstrated no tumors in all 4 animals observed for 6 months after 4 million URO-tsa cells were implanted. Compared with PC3 cells, URO-tsa cells demonstrated low constitutive and radiation-induced NF- $\kappa$ B activity. Studies herein further demonstrate that proteasome inhibition had no significant effect on clonogenic survival or NF- $\kappa$ B activity after radiation in URO-tsa cells.

Specific mechanisms of NF- $\kappa$ B activation by radiation are currently a rapidly expanding field of research. Ionizing radiation has been demonstrated to induce NF- $\kappa$ B via phosphorylation of serine residues 32 and 36 of I $\kappa$ B $\alpha$  by I $\kappa$ B kinase, but ultraviolet light leads to the activation of NF- $\kappa$ B without phosphorylation at the same residues. However, both ionizing and ultraviolet radiation-induced NF- $\kappa$ B activation was inhibited by proteasome inhibition (20). U251 glioblastoma cells were treated with radiation, and cellular fractions were isolated to evaluate the effects of MG-132 on I $\kappa$ B $\alpha$  degradation. Results demonstrated that MG-132 inhibited degradation of the insoluble subcellular fraction plasma membrane associated I $\kappa$ B $\alpha$  leading to the inhibition of radiation-induced NF- $\kappa$ B activity, suggesting that cellular localization of signaling pathways is important in radiation-induced NF- $\kappa$ B activation (21). It is of further note that NF- $\kappa$ B is composed of multiple subunits with mechanisms of regulation and effects of specific induction that are currently an active area of evolving research. Specific mechanisms of radiation-induced NF- $\kappa$ B activation and tumor survival will require thorough investigation of signaling pathways, cellular localization, and specific NF- $\kappa$ B target genes.

The potential implications of results derived from the present study are significant. Results demonstrated that prostate cancer cells can be selectively sensitized with associated inhibition of NF- $\kappa$ B activity using proteasome inhibition without affecting surrounding urothelial tissue. The inclusion of proteasome inhibitors in the treatment of prostate cancer may be a means to increase the therapeutic advantage of radiation therapy with respect to prostate cancer over normal adjacent tissues. Currently, great efforts are being made on the development of various forms of conformal radiation methods to increase the given dose to the tumor, while limiting the radiation dose to adjacent structures. The study herein is the first to demonstrate in a culture system that manipulation of NF- $\kappa$ B via proteasome inhibition coupled with radiation can achieve preferential sensitization of a tumor cell compared to a normal anatomically adjacent tissue. This combination of radiation and proteasome inhibition yields the same desired clinical outcome as conformal radiation therapy. Furthermore, the manipulation of NF- $\kappa$ B by methods such as proteasome inhibition could be coupled with conformal radiation delivery, thereby potentially improving the therapeutic advantage of both modalities.

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