Published in final edited form as:
Mol Cancer Ther. 2008 August ; 7(8): 2367–2376. doi:10.1158/1535-7163.MCT-08-0238.

SN52, a novel nuclear factor-κB inhibitor, blocks nuclear import of RelB:p52 dimer and sensitizes prostate cancer cells to ionizing radiation

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Abstract

The activation of nuclear factor-κB (NF-κB) is thought to protect cancer cells against therapy-induced cytotoxicity. RelB, a member of the NF-κB family in the alternative pathway, is uniquely expressed at a high level in prostate cancer with high Gleason scores. Here, we show that ionizing radiation (IR) enhances nuclear import of RelB, leading to up-regulation of its target gene, manganese superoxide dismutase (*MnSOD*), and renders prostate cancer cells resistant to IR. To selectively block RelB nuclear import, we designed a cell-permeable SN52 peptide, a variant of the SN50 peptide that has been shown to block nuclear import of NF-κB family members in the classic pathway. Inhibition of IR-induced NF-κB activation by SN50 and SN52 was achieved by selectively interrupting the association of p50 and p52 with nuclear import factors importin-α1 and importin-β1. Importantly, SN52 seems to be more efficient for radiosensitization of prostate cancer cells at clinically relevant radiation doses and has less cytotoxicity to normal prostate epithelial cells compared with the toxicity observed with SN50. These results suggest that targeting the alternative pathway is a promising approach to selectively radiosensitize prostate cancers and that SN52 may serve as a prototype biological agent for sensitizing prostate cancers to clinically relevant doses of IR.

Introduction

The transcription factor nuclear factor-κB (NF-κB) family, including RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100), regulates expression of numerous genes involved in immune response, inflammation, cell survival, and cancer (1–3). A Rel homology domain conserved in all NF-κB members is responsible for their dimerization, nuclear translocation, and DNA binding (4). Precursors of p105 and p100 are posttranslationally processed to DNA binding subunits, p50 and p52 (5,6), which form dimers with other NF-κB members for the activation of target genes (7,8). The NF-κB dimers are retained as inactive forms in cytoplasm by inhibitors that mask the nuclear translocation sequences (NLS) found in the Rel homology domain (9–11). In mammals, two distinct NF-κB signaling pathways exist to respond to different stimuli (12). The classic pathway, mediated by the RelA:p50 dimer and stimulated by cytokines, including tumor necrosis factor-α (TNF-α) and infectious agents, is

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

dependent on the degradation of IκBs. IKKβ, a major kinase component, phosphorylates IκBs to trigger ubiquitin-based degradation, leading to the release of the RelA:p50 dimer (13,14). The alternative pathway mediated by the RelB:p52 dimer is dependent on IKKα-activated processing of p100 and release of p52; it was identified by its response to other members of the TNF family with the exception of TNF-α (6,11,15). Although the alternative pathway is necessary for activation of the multiple target genes involved in secondary lymphoid organogenesis (15,16), its function in regulating cellular processes in response to internal and external factors remains largely unknown.

It has been well documented that NF-κB plays a critical role in coordinating the innate and adaptive responses that maintain cellular defense systems. Insights obtained from the study of NF-κB functions in cancer have provided a mechanistic link between inflammation and tumorigenesis and valuable information about NF-κB–mediated cytoprotection against cancer therapeutics (2,3,17,18). Consequently, inhibited NF-κB is being considered as a target for anticancer therapy. In fact, studies focused on the classic pathway have shown that suppression of p65 and ablation of IKKβ increase the chemosensitivity and radiosensitivity of some cancer cells (19,20). However, because the classic pathway is also known to play an important role in protecting normal tissues against chemotherapies and radiotherapies, the overall benefits from suppression of RelA for sensitization of cancer treatments remain to be investigated.

Prostate cancer is the second leading cause of cancer death in North American men despite rapid changes during the past two decades that include development and widespread use of prostate-specific antigens (21). Prostate-specific antigen screening gives us the ability to diagnose the disease in early stages and has resulted in an increase in the number of men diagnosed with prostate cancer (22). Current treatment strategies for locally confined prostate cancer include radiation therapy and surgical prostatectomy. Radiation results in lower disease-free survival rates when high-risk factors, such as increased PSA, elevated Gleason scores, and advanced T-stage, are present (23). Consequently, to improve the efficiency of localized radiation therapy when high-risk factors are present, strategies are needed to enhance the radiation sensitivity of prostate cancer cells. In many types of cancer, the RelA-based classic pathway is thought to play an important role in response to chemotherapeutics and radiotherapeutics; however, in prostate cancer with high Gleason scores, RelB is uniquely expressed at a high level (24). Thus, RelB may contribute to the radioresistance of high-risk prostate cancers. We previously showed that RelB is rapidly induced by radiation in prostate cancer cells and that selective inhibition of RelB significantly enhances radiosensitivity of aggressive prostate cancer cells (25,26). In the present study, we found that radiation activates the nuclear import of RelB, and we designed a cell permeable peptide, SN52, to block the nuclear import of the RelB:p52 dimer. The results of cytotoxicity analysis indicate that SN52 has a strong radiosensitization effect on prostate cancer cells with a low level of toxicity to normal prostate cells compared with SN50, which blocks nuclear import of the RelA:p50 dimer. This is the first study to show that the proof-of-concept use of a small peptide to inhibit the NF-κB alternative pathway is efficient for the improvement of radiation therapy of prostate cancer.

Materials and Methods

Cell Culture and Treatments

Human prostate epithelial prostate epithelial cells (PrEC; Cambrex Corp.) and human prostatic adenocarcinoma PC-3 (American Type Culture Collection) were grown and maintained in the recommended medium. The cultured cells were treated with ionizing radiation (IR) at a dose of 0.5 to 6 Gy using a 100-kV X-ray machine (Faxitron X-ray Corp.). To inhibit IR-induced NF-κB activation, the cells were treated with NF-κB inhibitory peptides (Celtek Bioscience LLC) at concentration of 0 to 60 μg/mL for 1 h before irradiation or 1 and 9 h after irradiation.

Cell Survival Analysis

Colony formation assay was used to quantify cytotoxicity of prostate cancer cells induced by treatments. The cells were plated in six-well plates at low densities. After overnight culture, the cells were treated as described above. The treated cells were cultured until colonies formed. The colonies were washed with 1× PBS and stained with a crystal violet dye. The surviving fraction was calculated as the ratio of the number of colonies formed to the number of cells efficiently plated. Trypan blue exclusion assay was used to determine the toxic effects of treatment on normal PrECs. The cells were plated at a concentration of 10^5 per well. After treatment, the cells were cultured for 5 days, stained with a 0.4% trypan blue dye, and counted using a Vi-cell cell viability analyzer (Beckman Coulter).

Immunocytochemistry

PC-3 cells (10^4) were seeded onto BD Falcon Culture Slides (BD Biosciences) with 300 μL of medium. After overnight culture, the cells were pretreated with the peptides at a final concentration of 40 mg/mL for 1 h and then treated with 6 Gy IR. Ten minutes and 12 h after IR treatment, the cells were fixed in 4% paraformaldehyde for 15 min. After washing twice with 1× PBS, the cells were blocked with 3% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at 37°C and then incubated with the primary antibodies against p50, p52, RelA, and RelB at 2 μg/mL in the donkey serum for 1 h at room temperature. After washing thrice with 1× PBS, the cells were further incubated with donkey anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories) at 7.5 μg/mL in the donkey serum for 1 h. After washing twice with 1× PBS and once with dH_2O, the cells were stained with Hochest (Invitrogen) at 20 μg/mL for 10 min and subsequently washed once with 1× PBS and once with dH_2O. The slides were allowed to dry and were viewed under laser scanning confocal microscopy (Leica Biocro-systems). To calculate the percentages of nuclear import of the NF-κB family members, densities of tested proteins in nuclei were measured from multiple areas of the slides using imaging quantification software incorporated in the confocal microscopy.

NF-κB Binding Assay

Cytoplasm and nuclei in the treated or untreated PC-3 cells were separated using a Nuclear Extraction Kit (Active Motif). The nuclear extracts were used to determine DNA binding activities of five members of the NF-κB family using an ELISA-based Trans NF-κB Family Kit (Active Motif). The experimental procedures were done according to the manufacturer's instructions.

Real-time PCR

mRNA was isolated from the treated and untreated PC-3 cells using a Micro-FastTrack 2.0 mRNA Isolation Kit (Invitrogen Corp.) and then analyzed using a SuperScript First-Strand Synthesis System for real-time PCR (Invitrogen Corp.) with gene-specific primers. Real-time PCR was done with a LightCycle System (Roche Molecular Biochemicals) according to the manufacturer's protocol. The primers for amplification of the human *RelA* and *RelB* genes were purchased from Santa Cruz Biotechnology. Primers for manganese superoxide dismutase (*MnSOD*) were as follows: forward, 5′-AGCATGTTGAGCCGGGCAGT-3′, and reverse, 5′-AGGTTGTTCACGTAGGCCGC-3′; for *β-actin*: forward, 5′-TGATGATATCGCCGCGCTCGTCGT-3′, and reverse, 5′-CACAGCCTGGATAGCAACGTACAT-3′.

Western Blots

To quantify the levels of the NF-κB members and the NF-κB target genes, 20 μg nuclear extracts and 100 μg cellular or cytoplasmic extracts were fractionated by a SDS-PAGE, 8% (w/v) polyacrylamide gel, and then transferred onto a nitrocellulose membrane and blotted

with primary antibodies to the NF-κB family members, MnSOD, β-actin, importin-α1, and importin-β1. With the exception of the MnSOD antibody obtained from Upstate Biotechnology, all primary antibodies and secondary antibodies were purchased from Santa Cruz Biotechnology. Three secondary antibodies were used: rabbit anti-goat IgG-horseradish peroxidase conjugated for detection of importin-α1 and importin-β1, goat anti-mouse IgG-horseradish peroxidase conjugated for detection of β-actin, and goat anti-rabbit IgG-horseradish peroxidase conjugated for detection of all other proteins. Western blots were visualized by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotechnology).

Immunoprecipitation

Immunoprecipitation was carried out by overnight incubation of 200 μg cytoplasmic extracts isolated from the treated and untreated PC-3 cells with 1 μg p50 and p52 antibodies at 4°C. Subsequently, 20 μL of protein A/G agarose (Santa Cruz Biotechnology) were added to the mixtures and incubated for 4 h at 4°C. Immunocomplexes were precipitated by centrifugation at 2,500 rpm for 5 min at 4°C. The supernatants were collected and the pellets were washed four times with radioimmunoprecipitation assay buffer [9.1 mmol/L Na_2HPO_4, 1.7 mmol/L NaH_2PO_4, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% v/v NP40, and 1% SDS (pH 7.2)] and resuspended in 20 μL of 2× sample loading buffer. The supernatants and pellets were fractionated by the SDS-PAGE gel and blotted with primary antibodies to importin-α1 and importin-β1.

Chromatin Immunoprecipitation

A ChIP-IT system (Active Motif) was used to quantify IR-induced NF-κB binding to the enhancer region of the *sod2* gene, according to the manufacturer's protocol. Chromatin isolated from the IR-treated PC-3 cells was pulled down using a p52 antibody, and the NF-κB enhancer region of the *sod2* gene was quantified by PCR. PCR primer sequences were as follows: upper strand, 5′-CGGGGTTATGAAATTTGTTGAGTA-3′ lower strand, 5′-CCACAAGTAAAGGACTGAAATTAA-3′. An exon2 fragment of the gene was amplified as an untargeted control. Primer sequences were as follows: upper strand, 5′-TGACCGGGCTGTGCTTTCTCG-3′; lower strand, 5′-ACTGCCTCCCGCCGCTCAGCC-3′. Western blots were done to quantify RelB in the chromatin immunoprecipitation preparations.

Statistical Analyses

Multiple independent experiments were done for each set of data presented. Data from PCR, real-time PCR, and Western blots were quantified using the imaging quantitative software Quantity One (Bio-Rad). Statistical significances between treatments and controls were analyzed using one-way ANOVA and Tukey's multiple comparison test followed by data analysis with GraphPad Prism version 4.0. Differences in the comparison tests lower than $P < 0.01$ were considered to be insignificant.

Results

IR Induces NF-κB Nuclear Import and DNA Binding

To elucidate how IR induces the activation of the NF-κB pathway, IR-treated PC-3 cells were stained with RelA and RelB antibodies to determine the influence of IR on their nuclear import. After irradiation, the RelA and RelB in cytoplasm and nucleus were observed at various times by confocal microscopy. The confocal images shown in Fig. 1 indicate that both RelA and RelB rapidly increased in the nuclei after IR. RelA level in the nucleus was enhanced throughout the times examined (Fig. 1A). RelB nuclear import was observed 10 minutes after

IR treatment, but declined at 1 hour from the level at 10 minutes and increased again until 12 hours after radiation (Fig. 1B). No significant changes in RelA and RelB nuclear levels were observed in the untreated controls in any of the examination times. The images of RelA and RelB in nuclei were quantified by analyzing multiple images from three independent experiments. The percents of imported RelA and RelB nuclei are represented in the right panels of graphs in Fig. 1A and B. To confirm the results of immunocytochemistry, cytoplasmic and nuclear extracts were quantified by Western blotting with RelA and RelB antibodies. The levels of RelA and RelB in nuclear extracts were increased by IR. In addition, the levels of RelA and RelB in the cytoplasmic extracts were also increased by IR, indicating that IR stimulates production of NF-κB family members in PC-3 cells (Fig. 1C). Consistent with the results of nuclear import, the DNA binding activities of members of the NF-κB family, with the exception of c-Rel, were increased by IR. Importantly, IR enhanced the DNA binding activity of RelB more than that of RelA (Fig. 1D). These results indicate that increased RelB nuclear import may play an important role in protecting against IR in prostate cancer cells.

Activation of RelB Leads to the Induction of MnSOD

We previously showed that IR rapidly induces MnSOD expression (25,26) and that an intronic NF-κB enhancer element is essential for transcriptional induction of the *sod2* gene stimulated by TNF-α and interleukin-1β (27). To determine if RelB is involved in the transcriptional induction of the *sod2* gene, chromatin immunoprecipitation assay was done to verify that RelB directly binds to the *sod2* enhancer element. Chromatin was precipitated using p52 antibody and quantified by PCR with gene-specific primers. As shown in Fig. 2A, amounts of *sod2* enhancer region and RelB nuclear level were similarly increased by IR at the same times. An untargeted control and a negative antibody control were included to ensure specificity of interaction between RelB and the *sod2* enhancer element. IR had no effect on the *GAPDH* promoter that was pulled down by the TFIIB antibody. Total RNA and proteins isolated from the IR-treated cells were quantified by real-time PCR with gene-specific primers and by Western blots with protein-specific antibodies. The results show that MnSOD was increased by IR at the RNA (Fig. 2B) and protein (Fig. 2C) levels.

NF-κB Inhibitory Peptides Block RelA and RelB Nuclear Import

SN50, a cell-permeable peptide containing the p50 NLS, was designed to block nuclear translocation of RelA:p50 dimer (28). To selectively block the nuclear import of RelB:p52, we designed a SN52 peptide by replacing the p50 NLS with the p52 NLS that is essential for processing of p100 (29). Two mutant peptides (SN50M and SN52M) were designed by changing the two positively charged residues lysine and arginine to the uncharged residues asparagine and glycine (Fig. 3A). The peptides were applied to the PC-3 cells for 1 hour before IR treatment. The effects of the peptide pretreatment on nuclear import of the NF-κB family members were first examined by immunolocalization. SN50 strongly blocked the IR-induced nuclear import of p50 and RelA and also exerted a minor effect on IR-induced RelB nuclear import (Fig. 3B). In contrast, SN52 selectively and completely blocked the IR-induced nuclear import of p52 and RelB without any effect on RelA (Fig. 3C). Consistent with the nuclear translocation, results of NF-κB binding assays show that SN50 inhibited the DNA binding activities of p50, RelA, and RelB, whereas SN52 only inhibited the binding activities of p52 and RelB (Fig. 3D). These results indicate that SN52 inhibits NF-κB function by selectively blocking the alternative pathway.

SN52 Selectively Blocks the Alternative Pathway through Competing with p52 for Nuclear Import Proteins

To confirm the effects of SN50 and SN52, levels of the NF-κB members in the nucleus and the expression levels of *sod2* target gene in cytoplasm were quantified. The results in Fig. 4A

show that IR induces activation of both classic and alternative pathways, resulting in an increase in MnSOD expression, which was abolished by pretreatment with SN50 and SN52. Interestingly, when the cells were pretreated with SN52, MnSOD expression was further reduced following IR compared with the unradiated controls, suggesting that combining SN52 with IR may suppress the constitutive level of NF-κB activity. To determine whether SN50- or SN52-mediated inhibition of NF-κB occurs as a result of its competition with p50 or p52 for nuclear import factors, cytoplasmic extracts from the peptide-treated cells were precipitated using p50 or p52 antibody. Supernatants and pellets were blotted with antibodies to the known nuclear import factors, importin-1α or importin-1β, respectively. As shown in Fig. 4B, IR enhanced the interaction between the importin proteins and p50 or p52. As expected, both SN50 and SN52 competed with p50 and p52 for the nuclear import proteins, leading to a blockade of nuclear translocation of NF-κB members, whereas neither SN50M nor SN52M had any competitive effects on NF-κB nuclear import. These results suggest that importin-1α and importin-1β serve as common factors in the nuclear import complex that is required for the nuclear translocation of distinct members of the NF-κB family. It has been reported that SN50 has an overtarget effect on inhibiting nuclear translocation of other transcription factors, such as activator protein-1, nuclear factor of activated T cell, and signal transducer and activator of transcription 1 (30). To further detect the overtarget effect of SN50, the influence of SN50 on Sp1 nuclear translocation in PC-3 cells was examined. The cytoplasmic extracts were pulled down by Sp1 antibody and blotted with the importin-1α or importin-1β antibody. The levels of both importin-1α and importin-1β were reduced in the SN50-treated cells compared with the untreated and the mutated peptide-treated controls. Importantly, no similar effect was observed in SN52-treated cells (Fig. 4C). In addition, similar levels of Sp1 protein were observed in the peptide-treated and untreated cells, indicating that the SN52 peptides have no effect on expression of the *Sp1* gene in the PC-3 cells. Consistent with the nonspecific effect of SN50, the nuclear level of Sp1 in the SN50-treated cells was lower than it was in the controls. These results indicate that, unlike SN50, SN52 has no overtarget effect on Sp1 nuclear translocation.

SN52 Enhances Radiosensitivity of Prostate Cancer Cells but Has Only Minor Toxic Effect on Normal Prostate Cells

Inhibition of the NF-κB classic pathway has been shown to be an efficient adjuvant approach to sensitize cancer cells to chemotherapy or radiotherapy by diminishing adaptive responses (19,20). In this study, the relative effects of SN50 and SN52 on radiosensitization of androgen-independent aggressive prostate cancer cells were tested using colony formation assay. PC-3 and DU-145 cells are resistant to low doses of IR, although a high dose of IR significantly kills the cells. Both SN50 and SN52 enhance the radiosensitivity. SN52 seems to efficiently radiosensitize PC-3 cells at a low dose range of between 1 and 2 Gy, but its radiosensitization effect is slightly lower than that of SN50 at the high dose range of between 4 and 6 Gy (Fig. 5A). On the other hand, the radiosensitization effect of SN52 in DU-145 is higher than SN50 at all of tested radiation doses (Fig. 5B). In addition, the toxicities of SN50 and SN52 to normal prostate epithelium, PrEC, were determined using trypan blue exclusion analysis. The PrEC cells were more sensitive to IR than the prostate cancer cells were, but the peptides tested exhibited different levels of cytotoxicity to the PrEC cells. SN50 showed significant toxicity to PrEC cells and even greater toxicity when combined with 2 Gy IR. In contrast to SN50, SN52 showed less toxicity to PrEC cells (Fig. 5C). To elucidate whether the difference in radio-sensitization of the tested cell lines is related to the presence of the NF-κB family members, the protein levels of five members of the NF-κB family were determined. PC-3 and DU-145 cells consistently expressed high levels of RelA, RelB, and c-Rel, which contain the activation domains required for transcriptional activation. Particularly, the expression of RelB in DU-145 cells is higher than its level in PC-3 cells, which consistently shows the radiosensitization effect of SN52 in cancer cells with a high level of RelB (Fig. 5D). In contrast to prostate cancer cells,

the PrEC cells expressed high levels of p50 and p52, but low levels of RelA, RelB, and c-Rel. The level of RelA is higher than RelB and c-Rel in the PrEC cells, indicating that inhibition of the classic pathway may cause toxicity to normal cells.

To determine the optimal concentration of peptides for radiosensitization, PC-3 cells were further treated with various concentrations of SN50 or SN52 before 2 Gy IR treatment. Although low doses of SN50 and SN52 are capable of enhancing IR-induced cell death, significant radiosensitization effects were observed when a final concentration of 40 μg/mL was used (Fig. 6A). No obvious additive effect was obtained with higher concentrations of peptides. Mutant peptides SN50M and SN52M had no apparent toxic effects. Based on the results of IR-induced RelB nuclear import (Fig. 1A), PC-3 cells were tested for optimal radiosensitization effect when peptide pretreatment was applied 1 hour before IR or 1 and 9 hours after IR. The results show that a high radiosensitization effect was obtained when peptide pretreatment was done 1 hour before IR (Fig. 6B). No additional effect was observed when peptides were applied twice, at 1 hour before and 9 hours after IR (data not shown). These results indicate that SN52, an inhibitor of the NF-κB alternative pathway, is efficient for enhancing radiosensitivity of prostate cancer cells at clinically relevant radiation doses and also can be conventionally applied before radiation treatment.

Discussion

Two major NF-κB pathways activate different sets of target genes involved in immune and inflammatory responses based on the nuclear translocation of RelA:p50 and RelB:p52 dimers (12). The target genes of the IKKβ–regulated classic pathway include cytokines, adhesion molecules, inflammatory mediators, inhibitors of apoptosis, and antioxidant enzymes (31–33). However, the target genes of the IKKα-regulated alternative pathway and related biological functions have not been fully elucidated. Our finding that radiation induces nuclear import of RelB leading to activation of prosurvival gene *sod2* suggests that this pathway may also regulate the cellular antioxidant system. We previously showed that TNF-α induces transcription of the *sod2* gene through RelA:p50 dimer binding to an intronic NF-κB element of the gene (27). Recently, we also found that RelB is able to bind to the intronic NF-κB element, leading to the induction of MnSOD in response to radiation (25), which is prevented by vitamin D3 in prostate cancer cells (26). The finding that MnSOD can be regulated by the two distinct NF-κB pathways is consistent with the observation that activation of the NF-κB target genesis modulated by exchange of dimers (34).

It is well documented that inhibition of NF-κB contributes to enhanced radiosensitization. SN50-based inhibition of the classic pathway has been shown to induce apoptosis and enhance chemosensitization and radiosensitization in cancer cells (35,36). Using SN52-containing NLS of p52 to selectively block the nuclear import of the RelB:p52 dimer, we have shown that SN52 efficiently blocks the alternative pathway without affecting the classic pathway. SN52 not only abolishes radiation-induced MnSOD but also reduces the constitutive level of MnSOD. Thus, for enhancing the sensitivity of prostate cancer cells to radiation using the same concentration of peptides, SN52-based inhibition of the alternative pathway is more efficient than SN50 inhibition of the classic pathway. Because SN50 is also able to partially block RelB nuclear import, the radio-sensitization effect of SN50 may include the inhibition of the classic pathway with a minor effect on the alternative pathway. Because the SN52 effect on radiosensitization is mainly based on inhibiting the alternative pathway, the evidence that SN52 is more toxic to prostate cancer cells than SN50 suggests that the alternative pathway is critical for radiosensitization of prostate cancer cells. The finding that SN52 selectively blocks the alternative pathway may also be useful for treating other types of cancer with a high level of RelB.

Although inhibition of the classic pathway may be a useful approach for enhancement of radiotherapies and chemotherapies, it may also sensitize normal cells to treatment. It has been shown that SN50 is toxic to normal tissues (37,38). The present study consistently shows that SN50 has a stronger effect on the ability of normal prostate cells to exclude trypan blue compared with SN52. In addition to the inhibition of the NF-κB classic pathway, the toxicity of SN50 may also be due to its effect on nuclear import of other transcription factors (30). The present study shows that SN50 is able to block Sp1 nuclear translocation. Together, these results suggest that the cytotoxicity of SN50 to normal cells may be caused in part by its overtarget effect. In addition, inhibition of the classic pathway may be toxic also to normal prostate cells. In contrast to SN50, SN52 has no inhibitory effect on the classic pathway and Sp1 nuclear translocation, and SN52-based selective blockade of the alternative pathway has a limited effect on normal prostate cells. Thus, the finding that SN52 efficiently enhances radiosensitivity of aggressive prostate cancer cells at low IR doses with limited effect on the growth of normal prostate cells provides a potential therapeutic approach for the treatment of prostate cancer with high Gleason scores.

In addition to verifying that increased nuclear RelB level is associated with its nuclear import, our findings also show that the reaction of nuclear importin proteins is blocked by SN52 targeting. The nuclear import of transcription factors requires an array of proteins, including importin-α and importin-β (initially identified as karyopherin-α and karyopherin-β). Importin-α is essential for the initial interaction with NLS of the imported proteins, and the importin-β functions with Ran, a small GTP-binding protein, to activate energy-dependent translocation (39,40). In the present study, we show that p50 and p52 antibodies pull down both importin-α1 and importin-β1 and that SN50 and SN52 are able to compete with p50 and p52 for the importin complex, leading to the blockade of RelA and RelB nuclear imports. However, because the molecular mechanism(s) of nuclear import of transcription factors remains largely unclear, the precise mechanism by which the importin proteins regulate nuclear import of RelB:p52 dimer in response to radiation will require further extensive investigation.

In summary, the present study shows that (α) inhibition of the NF-κB alternative pathway is more efficient for enhancing the effect of radiation on aggressive prostate cancers at the clinical dose being used in a standard fractionation protocol; (*b*) inhibition of the NF-κB alternative pathway is less toxic to normal PrECs; and (*c*) both classic and alternative pathways can activate common target genes, including *MnSOD*, in prostate cancer cells. Thus, the inhibition of the NF-κB alternative pathway may provide a unique opportunity for selective sensitization of prostate cancer to radiotherapy or chemotherapy.

Acknowledgments

Grant support: NIH grants CA49797 (D.K. St. Clair) and CA115801 (W.H. St. Clair).

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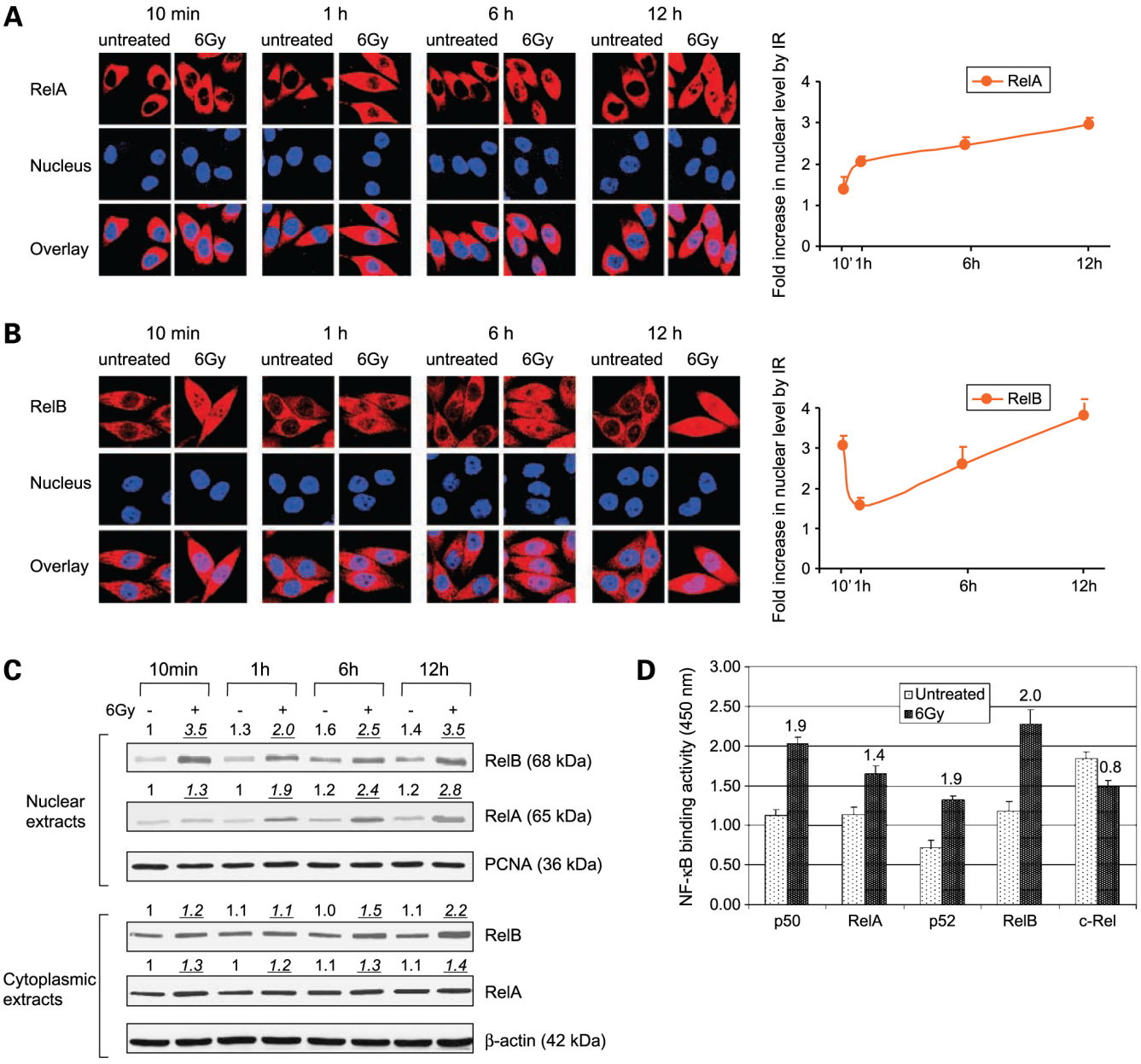

Figure 1. Nuclear levels of RelA and RelB induced by IR

PC-3 cells were treated with 6 Gy IR for the indicated times and stained with antibody against RelA (**A**) or RelB (**B**), respectively. The distribution of RelA and RelB between the cytoplasm and nucleus was determined by immunocytochemistry (*left*). Fold increases in nuclear levels of RelA and RelB at the indicated times were quantified by image analysis of multiple pictures obtained from three independent experiments (*right*). **C**, nuclear and cytoplasmic proteins were extracted from the IR-treated or untreated PC-3 cells and blotted with RelA or RelB antibody. The immunoblot signals were quantified and the effect of IR on the levels of RelA and RelB were normalized with the untreated controls and further normalized with loading controls (two-step normalization). **D**, 12 h after IR treatment, the nuclear extracts were subjected to a NF-κB binding assay kit. DNA binding activity of each member of the NF-κB family was determined by ELISA. Increases in the DNA binding activity were calculated by normalizing with the untreated controls. Fold increases or decreases in amounts of RelA or RelB levels in

nuclei (**C**) and DNA binding activity (**D**) compared with the untreated controls are indicated above corresponding bands or histograms, respectively.

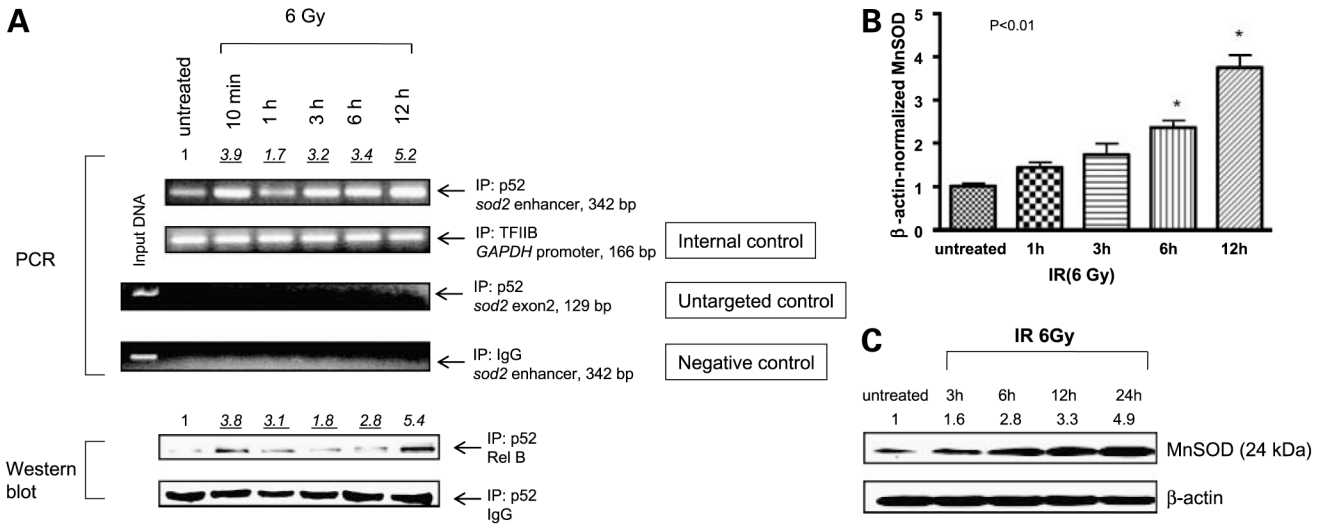

Figure 2. Induction of the NF-κB target *sod2* gene by IR

A, PC-3 cells were treated with 6 Gy IR and harvested at the indicated times after IR. Chromatin from the treated and untreated cells was precipitated using p52, TFIIB, or IgG antibody. The *sod2* enhancer region was analyzed by PCR (*top*). The *GAPDH* promoter region and an exon 2 fragment of the human *sod2* gene were amplified as a loading control and an untargeted control, respectively. IgG-precipitated product serves as a negative antibody control. RelB in the precipitated chromatin was measured by Western blots normalized with IgG (*bottom*). **B**, after IR treatment, mRNA was isolated and quantified by real-time PCR for the expression levels of MnSOD. *, significant difference compared with the untreated groups. **C**, MnSOD was quantified by Western blots. β-Actin was used as a loading control in **B** and **C**. Fold increases in DNA, mRNA, and protein in IR-treated groups compared with the untreated groups were calculated by the two-step normalization described in Fig. 1. The induction folds are indicated above the corresponding bands in **A** and **C**.

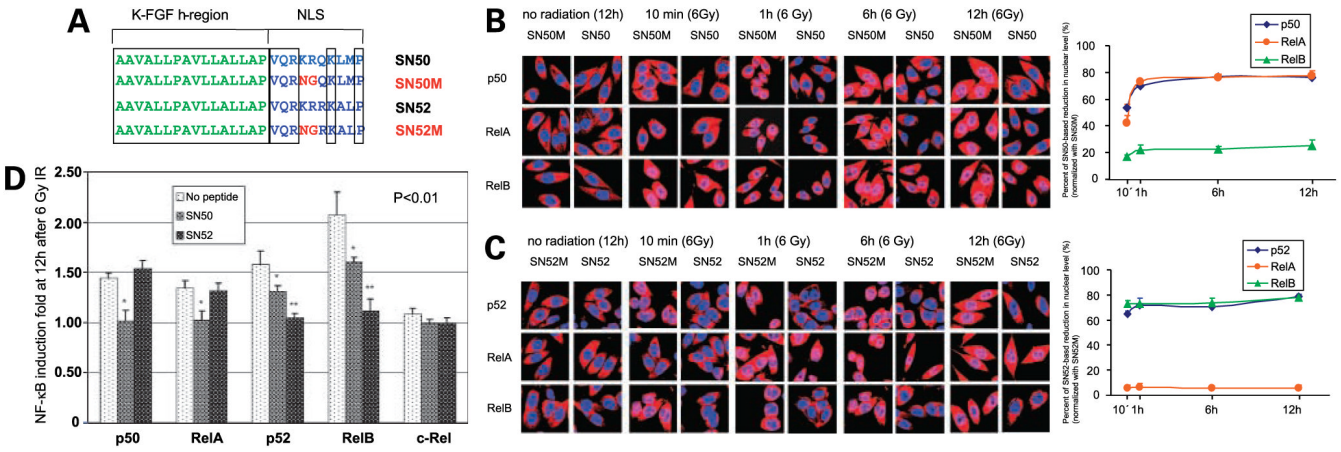

Figure 3. Inhibition of NF-κB nuclear import by SN50 and SN52

A, amino acid sequences of peptides used to inhibit NF-κB nuclear import. The KFGF h-region provides cell permeability, and NLS constitutes amino acids 360–369 residues of p50 or 335–344 residues of p52. Identical sequences are indicated by boxes. Two positive charged residues, lysine and arginine, were changed to uncharged residues, asparagine and glycine, in SN50M and SN52M. Inhibitory effect of SN50 (**B**) or SN52 (**C**) on nuclear import of NF-κB family members was determined by immunocytochemistry. Effect of SN50 or SN52 on nuclear import of NF-κB members was estimated by normalizing with SN50M or SN52M, respectively (**B** and **C**, *right panels*). PC-3 cells were pretreated with 40 μg/mL peptides for 1 h and followed by IR treatment at doses and times indicated. The cellular distribution of the NF-κB family members was detected using the corresponding antibodies. SN50M or SN52M was used as a negative control. **D**, 12 h after IR treatment, the inhibitory effect of the peptides on nuclear levels of the NF-κB family members was confirmed using DNA binding assay. * or **, significant differences in SN50-treated group or SN52-treated group compared with the untreated group, respectively.

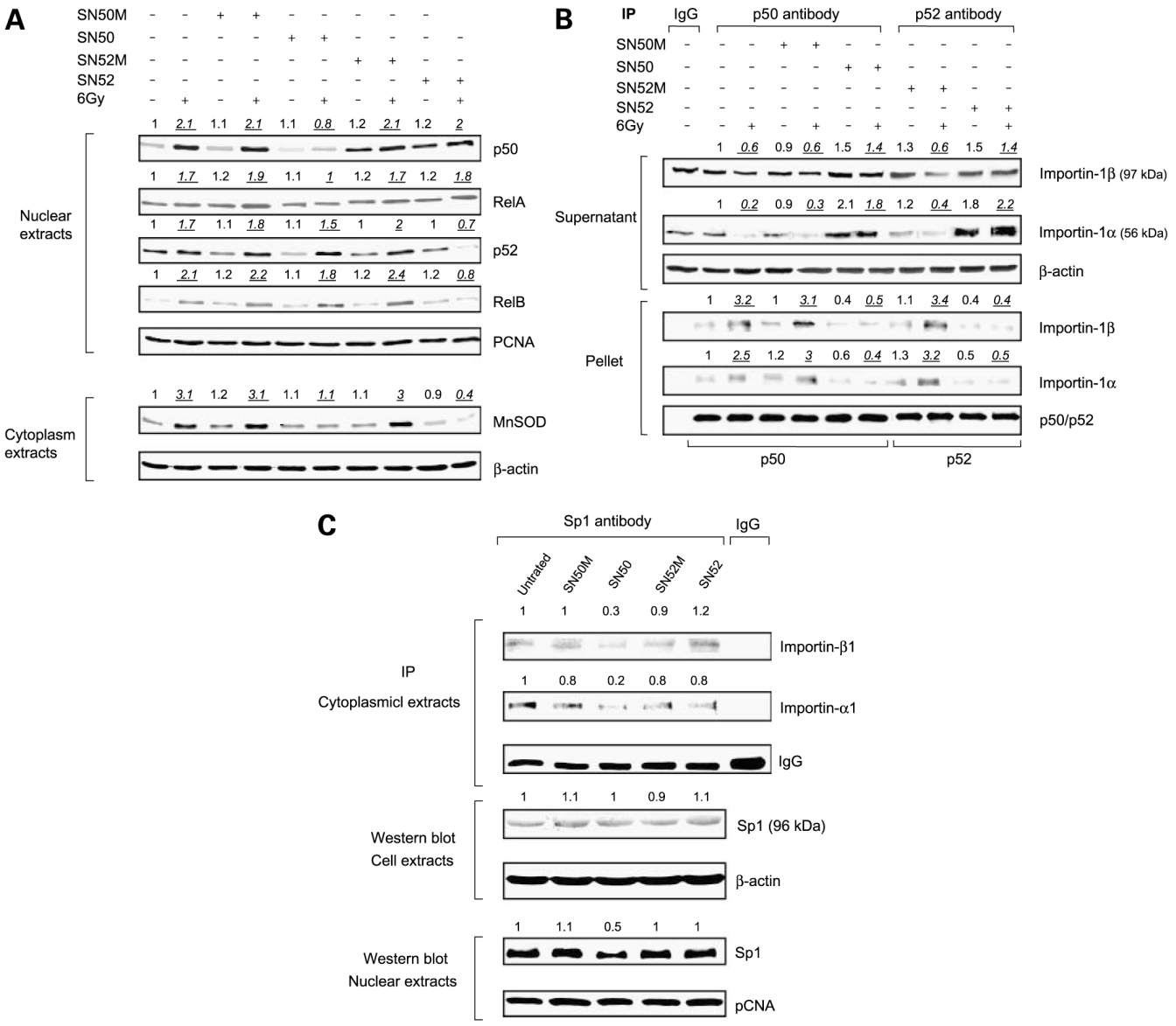

Figure 4. SN50- and SN52-based blockade of NF-κB nuclear import through competing for nuclear import proteins

PC-3 cells were pretreated with 40 μg/mL peptides for 1 h and followed by treatment of 6 Gy IR for 12 h. **A**, the levels of NF-κB members in the nucleus (*top*) and the resulting target gene levels in the cytoplasm (*bottom*) were determined by Western blots. **B**, cytoplasmic extracts were precipitated with p50 or p52 antibody. The supernatant fractions (*top*) and the pellet fractions (*bottom*) were blotted with importin-α1 or importin-β1 antibody. β-Actin, p50, and p52 were used as loading controls. **C**, the cytoplasmic extracts were pulled down by Sp1 antibody and blotted with importin-α1 or importin-β1 antibody. IgG was used as negative antibody control and loading control (*top*). Total cellular extracts were blotted with Sp1 antibody or β-actin antibody for loading control (*middle*). Nuclear extracts were blotted with Sp1 antibody or pCNA antibody for loading control (*bottom*). Fold increases or decreases compared with the untreated groups were calculated by the two-step normalization described in Fig. 1.

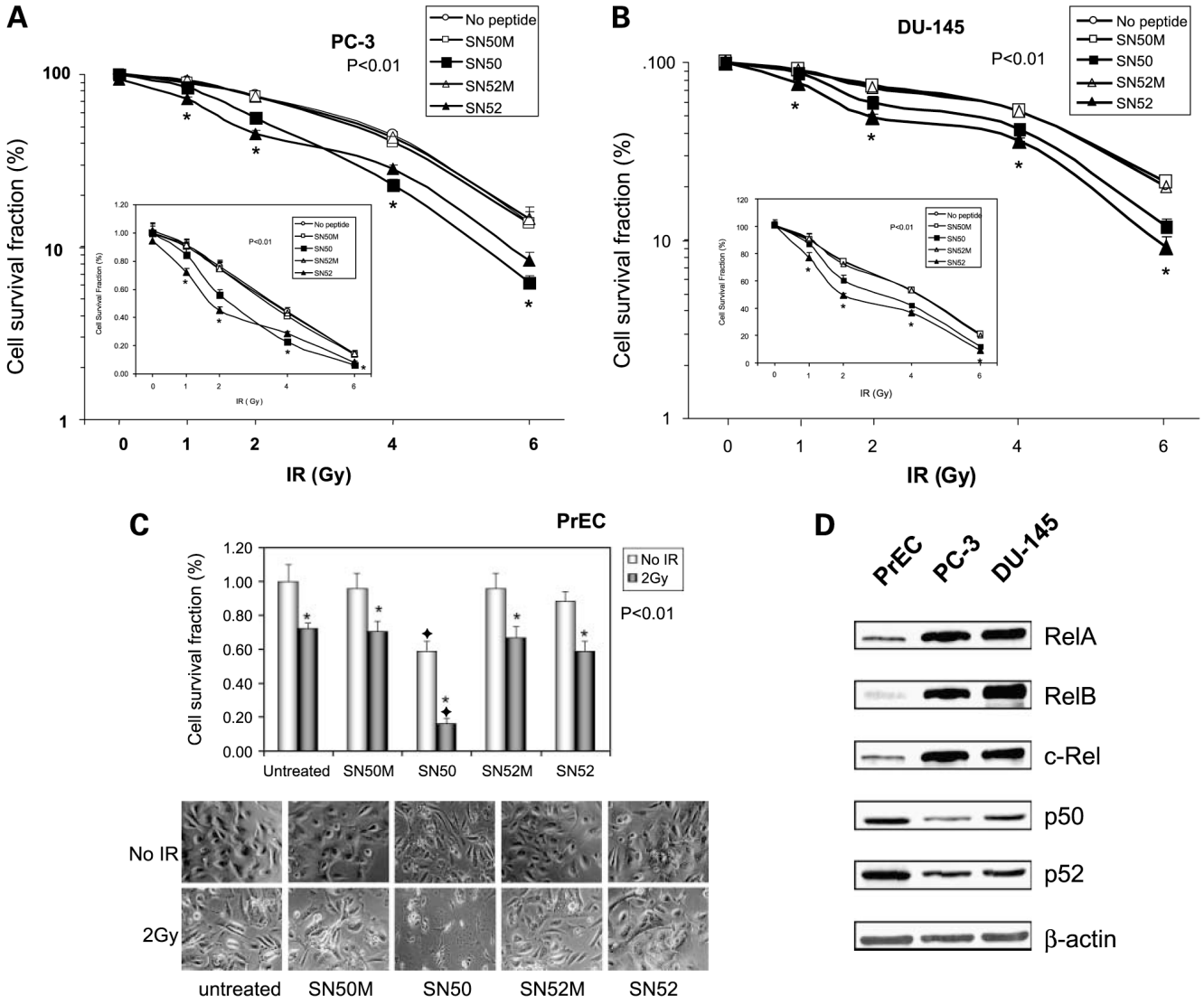

Figure 5. Cytotoxicity of SN50 and SN52 to prostate cancer and normal PrECs

A and **B**, PC-3 and DU-145 cells were treated with 40 μg/mL of each peptide and then treated with the indicated doses of IR. The effect of peptides on radiosensitivity was determined by colony formation assay. The results are plotted in a standard logarithmic scale as well as in a linear scale (*inset*) to clearly illustrate the combined toxic effects of each peptide with low doses of IR. *, significances for both SN50 and SN52 treatments compared with the results obtained with SN50M and SN52M. **C**, PrEC cells were treated with 40 μg/mL followed by 2 Gy IR treatment and cytotoxicity of treatments to the normal PrECs was quantified using trypan blue exclusion assay. *, significant differences compared with no IR treatment control; ♦, significant differences compared with no peptide treatment control. **D**, the constitutive levels of the NF-κB members in PrEC, PC-3, and DU-145 cells were measured by Western blots. β-Actin was used as a loading control.

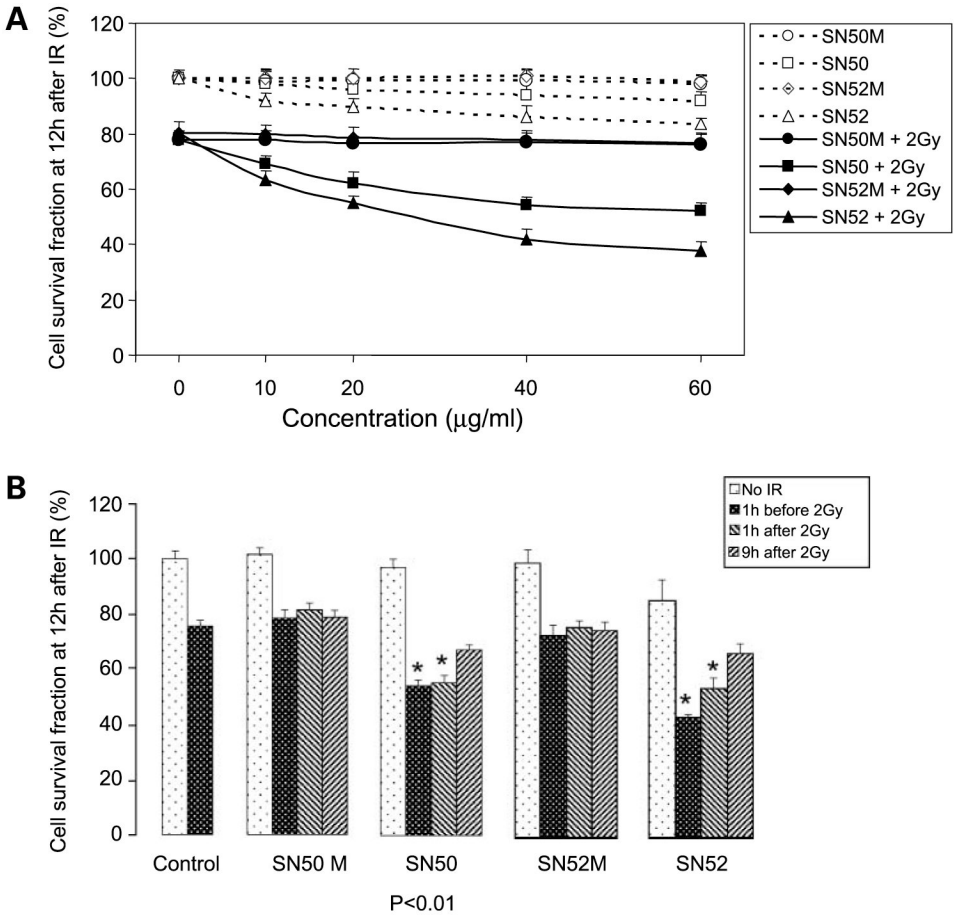

Figure 6. Examination of peptide treatment conditions

PC-3 cells were pretreated with each peptide at the indicated concentrations (**A**) and indicated times (**B**) before treatment with 2 Gy IR. Twelve hours after IR treatment, the effects of peptide concentration and treatment time were determined using colony formation assay. *, significant differences compared with no peptide treatment control.