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Characterization of an NBS1 C-Terminal Peptide That Can Inhibit Ataxia Telangiectasia Mutated (ATM)-Mediated DNA Damage Responses and Enhance Radiosensitivity[S]

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ABSTRACT

ATM and NBS1, mutation of which lead to the human autosomal recessive diseases ataxia telangiectasia and Nijmegen breakage syndrome (NBS), respectively, are essential elements in the cellular response to DNA damage induced by ionizing radiation (IR). ATM is a member of the phosphatidylinositol 3-kinase family and is activated by IR in an NBS1-dependent manner. The extreme C terminus of NBS1 contains an evolutionarily conserved sequence motif that is critical for binding to and activation of ATM after IR. ATM phosphorylates a series of targets to initiate cell cycle arrest and promote cell survival in response to DNA damage. Therefore, targeting the NBS1-ATM interaction may lead to a novel approach for specific ATM inhibition and radiosensitization. We developed small peptides containing the conserved C-terminal sequence of NBS1 to investigate whether these peptides can interfere with the DNA damage pathway. We found that wild-type NBS1 inhibitory peptides (wtNIP) can abrogate NBS1-ATM association in the presence or absence of IR. We also found that cells exposed to wtNIP displayed a significant reduction in radiation-induced γ-H2AX and NBS1 focus formation compared with cells treated with control peptides, demonstrating that wtNIP possesses a strong inhibitory effect on ATM. The inhibitory effect of wtNIP also leads to a significant decrease in clonogenic survival in response to IR. Furthermore, wtNIP does not radiosensitize cells with defective ATM, suggesting a specific inhibition of ATM. Together , these data provide a proof of principle for the use of NBS1 C-terminal small peptides as specific ATM inhibitors and radiosensitizers.

The DNA damage response is controlled by a concise series of signaling events that result in activation of cell cycle checkpoints, DNA repair, and apoptosis. This network is composed of a number of gene products, which include sensors, transducers, and effectors. DNA double-strand breaks (DSBs) are detected by sensor molecules that trigger the activation of transducing kinases. Transducers then amplify the signals by phosphorylation of effector molecules to regulate the signaling cascades that initiate cell cycle checkpoints, influence DNA repair machinery, or trigger apoptotic pathways. One central element in the network is the *ATM* gene, mutation of which contributes to the human autosomal recessive disorder ataxia-telangiectasia (A-T) (Shiloh, 2003). A-T is characterized by progressive neurodegeneration, variable immunodeficiency, an extremely high predisposition to the development of lymphoid malignancies, and a hypersensitivity to IR. Cells derived from patients with A-T show a variety of abnormalities, including cell cycle checkpoint defects, chromosomal instability, and hypersensitivity in response to IR. *ATM* is remarkable for its large size and the existence of a sequence in its carboxyl terminus similar to phosphatidylinositol 3-kinases. A family of genes, including *Tel1*, *Mec1*, and *Rad3* in yeast, *Mei-41* in *Drosophila melanogaster*, and *ATR* and *DNA-PK* in vertebrates, are similar in size and presence of the carboxyl terminal kinase sequence

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ABBREVIATIONS: DSB, double-strand break; A-T, ataxia-telangiectasia; IR, ionizing radiation; HEAT, huntingtin/elongation factor 3/the 65 kDa α-regulatory subunit of protein phosphatase 2A/yeast PI-3K TOR1; MRN, Mre11-Rad50-NBS1 complex; NBS1, Nijmegan breakage syndrome; ATM, ataxia telangiectasia mutated; DMEM, Dulbecco's modified Eagle's media; FBS, fetal bovine serum; NIP, NBS1 inhibitory peptide; wtNIP, wild-type NBS1 inhibitory peptide; scNIP, scrambled NBS1 inhibitory peptide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; SER, sensitizing enhancement ratio; ATR, ataxia telangiectasia related; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; siRNA, small interfering RNA; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; KU55933, 2-morpholin-4-yl-6 thianthren-1-yl-pyran-4-one.

and are all involved in controlling DNA damage response (Abraham, 2001). The functional domains of the ATM protein include several HEAT repeats that act as scaffolding for assembly of molecular components, a phosphatidylinositol 3-like kinase domain that can phosphorylate serine/threonine followed by glutamine (the S/T-Q consensus sequence), and a FAT carboxyl-terminal domain that may regulate protein activity and stability (Perry and Kleckner, 2003). ATM activation requires functional NBS1 (Cerosaletti and Concannon, 2004; Difilippantonio et al., 2005; Falck et al., 2005; Cerosaletti et al., 2006). Mutations in the *NBS1* gene are responsible for Nijmegen breakage syndrome (NBS), a hereditary disorder that imparts an increased predisposition to development of malignancy and a hypersensitivity to IR (Shiloh, 1997). NBS1 forms a complex with Mre11 and Rad50 to be called the MRN complex. MRN is highly conserved, and it influences each aspect of chromosome break metabolism (Varon et al., 1998). Studies have shown that the MRN complex can detect DNA double-strand breaks and recruit ATM to damaged DNA molecules (Lee and Paull, 2004, 2005). The C terminus motif of NBS1 contains a conserved sequence motif that binds to two of the HEAT repeats (2 and 7) of ATM. This interaction is essential to activate the kinase (Falck et al., 2005).

Because the binding of NBS1 is critical for ATM to be functioning in response to DNA damage, we hypothesized that interfering with the NBS1-ATM interaction may block ATM activation and confer radiosensitization. To test this hypothesis, we developed several small peptides containing the conserved C-terminal sequence motif of NBS1 and fused them to a polyarginine internalization sequence. Herein, we describe the characterization of the C-terminal NBS1 inhibitory peptide in terms of internalization, half-life, cellular cytotoxicity, effects on the DNA damage response, and radiosensitivity. Together, these data may lead to a better understanding of the mechanisms that could be used to increase the radiosensitivity of cancer and provide data that could be rapidly translated into the development of novel radiosensitizing drugs.

Materials and Methods

Cell Culture. Human tumor cell lines HeLa and DU-145 (American Type Culture Collection, Manassas, VA), and human simian virus-40 transformed fibroblast cell line GM9607 (Corriell Cell Repositories, Camden, NJ) were maintained in exponential growth in DMEM/10% FBS, in a 5% CO_2 humidified atmosphere. The glioma cell line M059J (Corriell Cell Repositories) were maintained in exponential growth in RPMI 1640 medium/15% FBS in a 5% CO_2 humidified atmosphere.

Peptide Synthesis. All peptides were synthesized by Abgent (San Diego, CA) and labeled with a biotin tag at their N termini for detection in vitro. Three peptides were produced: 1) one containing the polyarginine (R_9) internalization sequence alone, 2) a wild-type NBS1 inhibitory peptide (wtNIP) corresponding to amino acids 735 to 744 of human NBS1, and 3) a random sequence peptide in which amino acids 735 to 744 of human NBS1 were scrambled (scNIP). The peptides were dissolved in dimethyl sulfoxide, stored at −20°C, and reconstituted in DMEM/10% FBS before use.

Irradiation. An X-RAD 320 Irradiation Cabinet (Precision X-Ray, East Haven, CT) was employed at 320 kV and 160 mA, with a 0.8-mm Sn + 0.25-mm Cu + 1.5-mm Al (half-value layer ≅ 3.7 Cu) filter at a target-to-source distance of 20 cm and a dose rate of 3.4 Gy/min. All irradiations were conducted under normal atmospheric pressure and temperature.

Immunoprecipitation and Western Blotting. For coimmunoprecipitation of ATM, NBS1, and MRE11, cells were lysed for 1 h in ice-cold lysis buffer, which consisted of 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 5 mM Na_3VO_4, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation, supernatants were incubated with antibodies. After extensive washing with the lysis buffer, immunoprecipitates were analyzed by immunoblot using specific antibodies. For Western blotting analysis, samples (cell lysates or immunoprecipitates) were separated on to 2% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with various antibodies.

Immunofluorescence Microscopy. Exponentially growing cultures of cells were plated on sterile 22-cm^2 coverslips and incubated for 24 h at 37°C in 5% CO_2 humidified air before they were treated with the NIP peptides at room temperature. Coverslips were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde and 0.25% Triton X-100 for 15 min at room temperature, blocked for 30 min at room temperature, and incubated with fluorescein isothiocyanate-conjugated streptavidin or anti-γ H2AX and phospho-NBS1 antibodies (Rockland Immunochemicals, Gilbertsville, PA) for 1 h at room temperature. Coverslips were then mounted with Vectashield Elite (Vector Labs, Burlingame, CA) and observed with a Leica fluorescence microscope. Images were captured at 40× magnification using a Retiga EXi digital camera (QImaging, Surrey, BC, Canada) and analyzed with Image-Pro Plus software (ver. 4.1; Media Cybernetics, Inc., Bethesda, MD).

MTT Assay. For cytotoxicity studies, exponentially growing cultures of HeLa or DU-145 cells were harvested, plated in 96-well plates (5000 cells/well) in complete media, and incubated overnight. On the following day, cells were treated with the NIP peptides (0, 5, 10, 20, 50, or 100 μM) or paclitaxel (Taxol; 0, 10, 50 or 100 μM) as a positive control. At the end of the time course, an MTT cell viability assay (Promega Corp., Madison, WI) was used according to the manufacturer's guidelines to determine peptide cytotoxicity.

Colony Formation Assays. To determine radiosensitivity, the colony-forming assay was incorporated. Cells were harvested with 0.125% trypsin/0.05% EDTA, pelleted, and resuspended in 1 ml of fresh media with a 22-gauge needle to disperse clumps before hemocytometer counting in trypan blue. Cells were then plated at limiting dilutions in six-well plates and allowed to adhere overnight. Cultures were treated with phosphate-buffered saline, R_9, wtNIP, or scNIP for 1 h and irradiated (0–6Gy). Fresh peptides were added every 4 h until 24 h after IR, when the medium was replaced with peptide-free medium. Cultures were incubated for 1 to 2 days, harvested, and stained with 0.5% crystal violet in methanol. Colony number was determined with a dissecting microscope. A population of >50 cells was counted as one colony, and the number of colonies was expressed as a percentage of the value for untreated mock-irradiated control cells. The surviving curves were plotted by linear regression analyses, and the D_0 value represents the radiation dose that leads to 37% of survival. To determine the radiosensitizing potential of the peptides compared with other small-molecule inhibitors, we calculated the sensitizing enhancement ratio (SER) based on the dose of radiation required to reduce survival to 37% in the presence of scNIP or wtNIP. The following formula was used:

$$\text{SER} = \frac{D_0 \text{ for scNIP-treated cells}}{D_0 \text{ for wtNIP-treated cells}}$$

Statistics. To establish statistical significance, Student's t test was incorporated. The data were first fit to each experimental group over a dose range of 0 to 6 Gy. Significant differences were established at $p < 0.05$.

Results

Internalization and Cytotoxicity of the C-Terminal NBS1 Inhibitory Peptides. Previous studies have revealed that the C-terminal NBS1 domain is critical for its binding to ATM, and an NBS1 truncated derivative lacking the C-terminal 20 residues does not associate with ATM in vitro (Cerosaletti and Concannon, 2003, 2004; Falck et al., 2005; Cerosaletti et al., 2006). In addition, it has been shown that expression of an NBS1 transgene lacking the ATM binding domain in NBS cells leads to a dramatic reduction in ATM activation (Difilippantonio et al., 2005). Because inhibiting NBS1 association with ATM leads to suboptimal ATM activation after IR, the NBS1-ATM interaction can be a novel target for developing radiosensitizers. One approach to inhibiting NBS1-ATM interaction would be to use small peptides containing the conserved C-terminal sequence,which will presumably compete with endogenous NBS1-ATM interactions (Fig. 1A). Therefore, we designed peptides containing two functional domains: one an interfering domain that will inhibit the NBS1-ATM association, and the other an internalization domain that will transport the interfering peptides into cells. For the interfering domain, we used the amino acid sequences containing the conserved C-terminal motif of NBS1 as shown in Fig. 1B. This sequence contains the shortest ATM binding motif based on in vitro data (data not shown). For the internalization domain, we used a polyarginine sequence, which has been shown to have a significant efficiency of transporting small peptides and proteins across the plasma membrane (Fuchs and Raines, 2004; Deshayes et al., 2005) Three peptides were generated, including the R_9-alone, and a wtNIP corresponding to amino acids 73to 44 of human NBS1. The third peptide was designed as a negative control, using a random sequence generator to produce a peptide in which amino acids 735 to 744 of NBS1 were scrambled (scNIP). These peptides were labeled with a biotin tag at their N termini for detection in vitro.

We first evaluated the internalization of the fusion peptides. Treatment of HeLa cells with R_9, wtNIP, or scNIP at a concentration of 10 μM for 1 h led to a significant cellular uptake of peptide (Fig. 2). R_9, wtNIP, and scNIP internalization was localized to the cytoplasmic and nuclear compartments, whereas the control group, treated with DMEM alone, shows no fluorescent signal. Because the peptides would be used in radiation studies, we then determined the length of time the peptides remain in cells to ensure that the peptides would be present throughout the DNA repair process after IR. Cells treated with wtNIP or scNIP have significantly decreased fluorescence 8 h after treatment (Supplemental

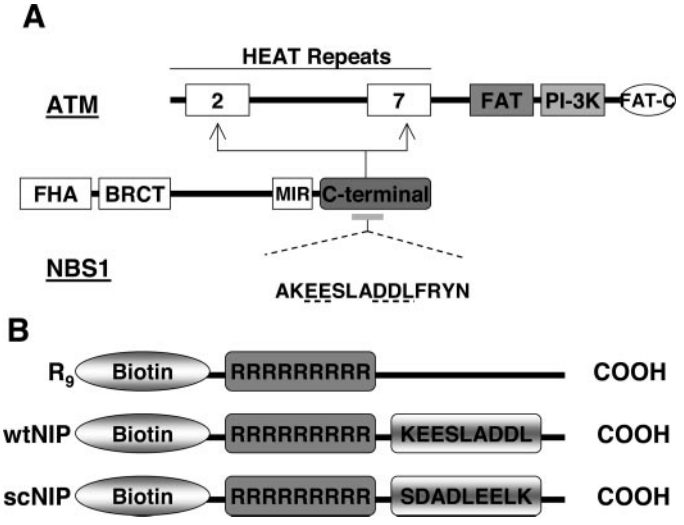

Fig. 1. Development of the NBS1 inhibitory peptides. A, schematic illustration of functional domains of ATM and NBS1 and their interaction. The C terminus of NBS1 is required for ATM activation and recruitment to sites of DNA damage. It consists of at least two sets of amino acid residues, 736 to 737 (EE) and 741 to 742 (DDL), that are evolutionarily conserved and necessary for ATM binding. NBS1 binds to two sets of the HEAT repeats [HEAT repeat 2 (amino acids 248–522) and HEAT repeat 7 (amino acids 1436–1770)] in ATM. B, the amino acid sequences for the R_9, wtNIP, and scNIP peptides developed.

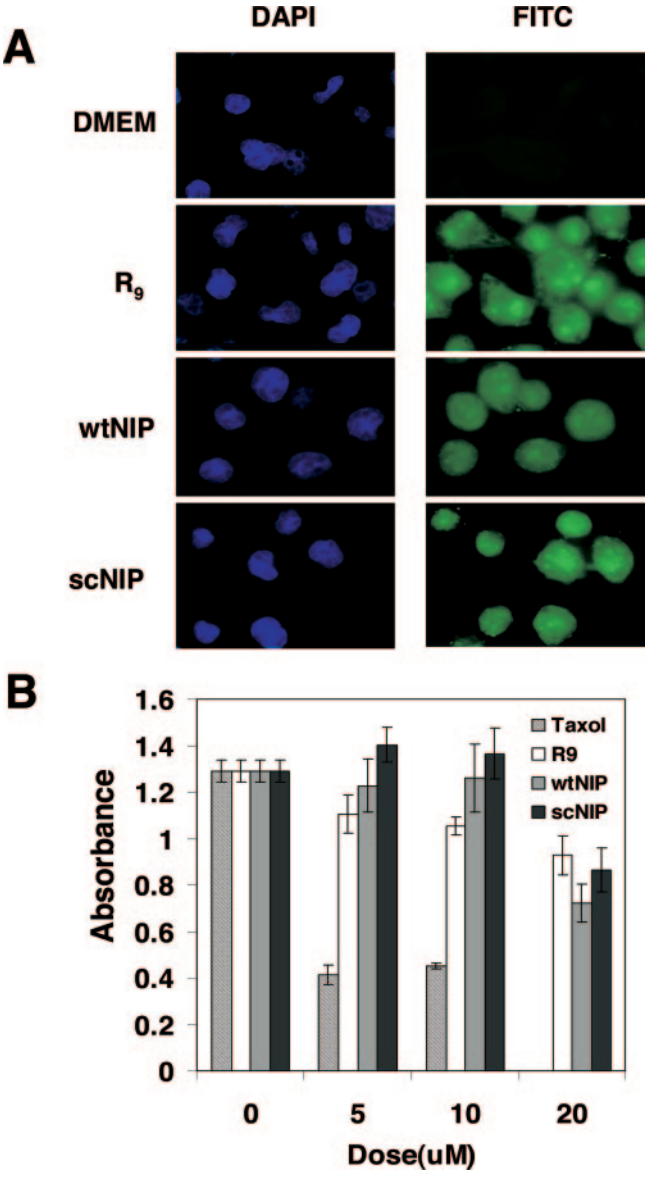

Fig. 2. Peptide internalization and cytotoxicity. A, HeLa cells were treated with 10 μM R_9, wtNIP, or scNIP for 1 h and analyzed by immunofluorescence microscopy after staining with fluorescein-conjugated streptavidin. B, HeLa cells were treated with paclitaxel and the NIP peptides at indicated doses. Twenty-four hours after treatment, cell survival was quantified by a standard MTT assay.

Fig. 1), suggesting that the NIP peptides should be added to cells every 4 to 6 h in the first 24 h after treatment with IR to achieve maximum inhibitory effects.

We then determined in vitro cytotoxicity of R_9, wtNIP and scNIP. HeLa cells grown in 96-well plates were treated with the peptides (0, 5, 10, 20, 50, or 100 μM) or paclitaxel (0, 10, 20, 50, or 100 μM) for 24 h. After treatment, the MTT assay was used to measure the production of solubilized formazan, a metabolic indicator of cell proliferation. The peptides demonstrated no growth inhibitory or cytotoxic effects up to 72 h after treatment (Fig. 2B), when the peptide doses were lower than 20 μM. Based on the cytotoxicity observed in the MTT assay, we chose 10 μM as the working concentration for all subsequent experiments. The effect of 10 μM R_9, wtNIP, and scNIP on clonogenic survival displayed no significant difference between treatment groups ($p < 0.05$) (Data not shown). It is noteworthy that dose and time course experiments have been preformed in several other cell lines, and our data confirmed rapid internalization and minimal cytotoxicity of these peptides (data not shown).

wtNIP Abrogated the NBS1-ATM Interaction. To investigate whether R_9-conjugated NIP peptides could inhibit NBS1-ATM interactions, we performed coimmunoprecipitation experiments in cells treated with the NIP peptides. Four hours after peptide treatment, HeLa cells were harvested and subjected to immunoprecipitation using an anti-NBS1 antibody. The immunoprecipitates were then blotted with anti-ATM, NBS1, and MRE11 antibodies. We observed a normal level of ATM-NBS1 association in R_9-treated cells compared with control cells. However, in wtNIP-treated cells, NBS1 was no longer able to bring down ATM (Fig. 3). Furthermore, the wtNIP affected only the NBS1-ATM interaction and did not interfere with NBS1 binding to MRE11. In contrast, scNIP did not affect the NBS1-ATM interaction. In cells treated with IR, wtNIP showed an effect similar to that in unirradiated cells. These observations demonstrate that wtNIP can abrogate the NBS1-ATM interaction in the absence or the presence of DNA damage.

wtNIP Inhibits IR-Induced γ-H2AX and NBS1 pSer343 Focus Formation. One of the earliest responses to IR-induced DNA damage is the formation of γ-H2AX foci, which requires functional ATM (Burma et al., 2001; Furuta et al., 2003). Because wtNIP showed an inhibitory effect on the NBS1-ATM interaction, we investigated whether IR-induced γ-H2AX focus formation was inhibited by the peptide. Immunofluorescence microscopy was used to detect the presence of γ-H2AX foci in mock-irradiated or irradiated cells in the presence of R_9, wtNIP or scNIP. The average number of γ-H2AX foci/nucleus in HeLa cells significantly increased after IR in cells treated with R_9 (42 foci/nucleus) or scNIP (41 foci/nucleus), whereas cells treated with wtNIP displayed only an average of 6.9 γ-H2AX foci/nucleus, similar to that of mock-irradiated cells (Fig. 4). Similar results were observed in DU-145 cells, whereas R_9 or scNIP exposure did not affect IR-induced focus formation, and wtNIP showed significantly reduced H2AX foci/nucleus (Supplemental Fig. 2). Therefore, IR-induced γ-H2AX focus formation can be inhibited by wtNIP.

To further support the idea that wtNIP can inhibit ATM-mediated DNA damage pathways, we investigated IR-induced NBS1 focus formation, an event considered to be an ATM-dependent process at the sites of DSBs (Lim et al., 2000). NBS1 foci are a result of ATM-mediated NBS1 phosphorylation on serine 343. Using an anti-phospho-Ser343 NBS1 antibody, we observed that NBS1 phosphorylation was significantly inhibited in cells treated with wtNIP compared with those treated with R_9 or scNIP (Fig. 5A and supplemen-

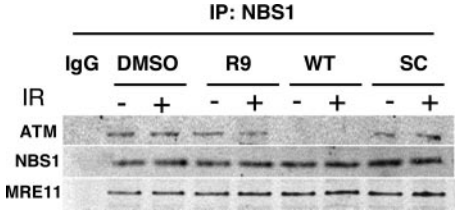

Fig. 3. wtNIP inhibits NBS1-ATM binding. HeLa cells treated with the NIP peptides were irradiated (0 or 6 Gy). Immunoprecipitation was performed with a rabbit NBS1 antibody, and Western blotting was performed with monoclonal antibodies against ATM, NBS1, or MRE11.

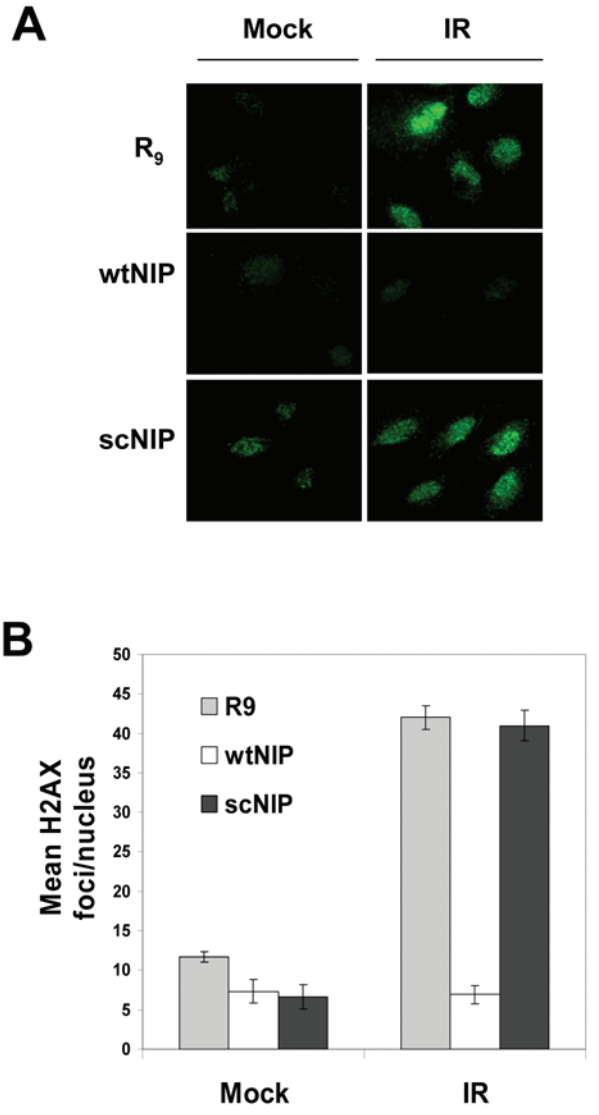

Fig. 4. WtNIP can inhibit γ-H2AX focus formation. A, HeLa cells were treated with 10 μM R_9, wtNIP, or scNIP for 1 h, irradiated with 0 or 6Gy, and harvested 30 min later before immunofluorescence microscopy was employed to detect radiation induced-γ -H2AX foci. B, the mean γ-H2AX nuclear foci per nucleus were determined for each image using Image-Pro Plus 5.1 software and is expressed in arbitrary units. Error bars represent ± 1 S.D.; graphed are the mean of three independent experiments.

tal Fig. 3A). The average number of foci in mock-irradiated HeLa cells was 6, 8, and 6 for R_9, wtNIP, and scNIP, respectively. Cells treated with R_9 or scNIP displayed 25 and 31 foci per nucleus, whereas cells treated with wtNIP showed only 6 foci per nucleus after treatment with 6-Gy IR (Fig. 5B).

It is important to note that there was a low level of background focus formation for both NBS1 and γ-H2AX phosphorylation, which has been correlated to mitosis in normally growing mammalian cell cultures (McManus and Hendzel, 2005).

wtNIP Increases Radiation Sensitivity. We then tested whether exposure to the NIP peptides will increase cellular radiosensitivity using the colony forming assay. Figure 6A depicts the survival curves for HeLa cells treated with R_9, wtNIP, or scNIP over a dose range of 0 to 6 Gy. We found that neither R_9 nor scNIP affects radiosensitivity, whereas wtNIP can significantly decrease IR-induced survival. Radiation survival curves were characterized based on D_0 to define the effect of NIP effect on radiosensitivity. D_0 represents the mean lethal dose required for 37% survival and is a measure of the intrinsic radiosensitivity of the cell. D_0 values for HeLa treated with wtNIP were 1.9 compared with 3.0 for cells treated with scNIP. To establish the statistical significance of wtNIP-induced radiosensitivity, Student's *t* test (paired two-sample for means) was incorporated. The data were first fit to each experimental group over a dose range of 0 to 6 Gy. Significant differences ($p < 0.05$) in clonogenic survival were observed between cells treated with wtNIP and those treated with DMEM, R_9, or scNIP. The SER was 1.58. This is comparable with other tested radiosensitizers, including gemcitabine, 5-fluorouracil, pentoxifylline, vinorelbine, and some ATM-specific radiosensitizers with SERs from 1.1 to 2.5 (Zhang et al., 1998; Lawrence et al., 2001; Robinson and Shewach, 2001; Strunz et al., 2002; Collis et al., 2003; Zhang et al., 2004). These observations have been confirmed in the prostate cancer cell line DU-145 (data not shown) with an SER of 1.46. Taken as a whole , they provide strong evidence for the radiosensitizing potential of the wtNIP peptide.

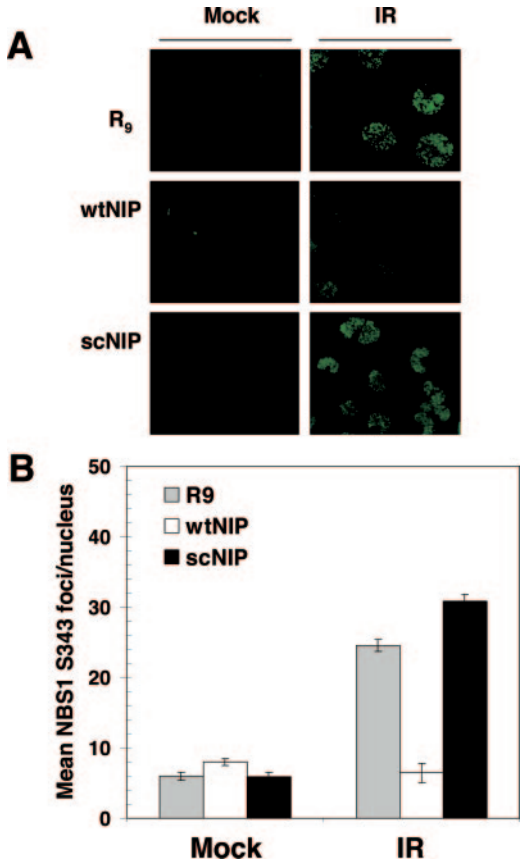

Fig. 5. Exposure to the wtNIP peptide abrogates IR-induced NBS1 phosphorylation. A, HeLa cells were treated with 10 μM R_9, wtNIP, or scNIP for 1 h, irradiated with 0 or 6Gy, and harvested 120 min later before immunofluorescence microscopy was employed to detect radiation induced-NBS1 focus formation using an anti-Ser343 NBS1 antibody. B, the mean number of NBS1 foci per nucleus was determined from a population of at least 25 cells in three independent experiments. Error bars represent ± 1 S.D.; graphed are the mean of three independent experiments.

Because wtNIP contains the conserved ATM binding sequence of NBS1, and this sequence is also conserved in the C terminus of ATR-interacting protein and KU80, the interacting proteins of ATR and DNA-PKcs, respectively, it was possible that it might also inhibit ATR or DNA-PKcs (Abraham, 2001). To test this possibility, we performed colony-forming assays in cell lines with defective ATM (GM9607) or DNA-PKcs (M059J). Although treatment with wtNIP led to an increase in radiosensitivity in M059J cells (Fig. 6C) with an SER of 1.83, GM9607 (Fig. 6D) displayed no change in radiosensitivity. Because GM9607 cells are ATM-deficient and have functional ATR and DNA-PKcs, our observations strongly suggest that wtNIP can specifically target ATM, but not ATR or DNA-PKcs, to achieve radiosensitization.

Discussion

Because ATM is central to cellular responses to irradiation, blocking its activation or activity could make any type of tumor much more sensitive to radiation. Since cloning the gene in 1995, investigators have employed several methods to develop specific ATM inhibitors. These methods include antisense RNA, small interfering RNA (siRNA), and screening of small molecule inhibitors of ATM. Subcloning a full-length cDNA of ATM in the opposite orientation into CB3AR cells significantly increased radiosensitivity (Zhang et al., 1998). The development of siRNA also led to the generation of an siRNA that could inhibit ATM function in prostate cancer cells. Both DU-145 and PC-3 cells, when transfected with these plasmids, exhibited an increase in radiosensitivity at clinically relevant radiation doses (Collis et al., 2003). More recently, the use of high-throughput screening has provided a new generation of ATM inhibitors that can be quickly translated to clinical studies. By screening a combinatorial library of compounds around the DNA-PKcs inhibitor LY294002, Hickson et al. (2004) reported a compound (KU55933) to selectively inhibit the ATM kinase. Their studies have shown a significant increase in radiosensitivity in HeLa cells. However, the in vivo radiosensitization effect and the toxicity of the compounds have not been reported.

Despite these promising findings, one of the major concerns of developing ATM inhibitors is the uncertainty of pleiotropic effects of such inhibitors. Due to the complex effects associated with malfunction of the protein kinase, the outcome of directly targeting ATM kinase activity can be complicated, in that it is unclear whether the only effect of these reagents will be to confer radiosensitization.

Instead of directly inhibiting the ATM kinase activity to

increase radiosensitivity, an alternative approach is to target IR-induced ATM activation, because this will directly lead to an increase in radiosensitivity without interfering with other important functions of ATM in the absence of DNA damage. Because the NBS1-ATM interaction is important for IR-induced activation of ATM, selectively disrupting the signaling pathway would be a novel approach for developing radiosensitizers. Furthermore, because the C-terminal of NBS1 association with ATM is necessary for ATM activation, we reasoned that a small peptide containing a portion of this conserved C-terminal domain (i.e., KEESLADDL) would compete with the NBS1-ATM association in vivo and sensitize tumor cells to radiation. Our data demonstrate that the wild-type NBS1 peptide can be used to inhibit ATM activation and induce radiosensitization.

Because the wtNIP peptide contains the conserved sequence among the phosphatidylinositol 3-kinase interacting proteins, such as ATR-interacting protein and Ku80 (Falck et al., 2005), we further reasoned that wtNIP could possibly interfere with ATR and DNA-PKcs activation. We tested the radiosensitizing effect of the peptides in cells with deficient ATM or DNA-PKcs. If the wtNIP could inhibit ATR or DNA-PKcs, then the ATM-deficient cells should be sensitized. However, the radiosensitivity of GM9607, which lacks ATM but has functional ATR and DNA-PKcs, was not affected by the peptide. In contrast, the DNA-PKcs mutant cells showed an increased radiosensitivity similar to that of HeLa and DU-145 cells treated with wtNIP. These observations therefore demonstrate specific ATM inhibition by the wtNIP peptide.

In summary, we have established a proof of principle in vitro, with results that may lend insight into a novel approach to the development of powerful radiosensitizers for clinical cancer therapy and use the peptides as specific ATM

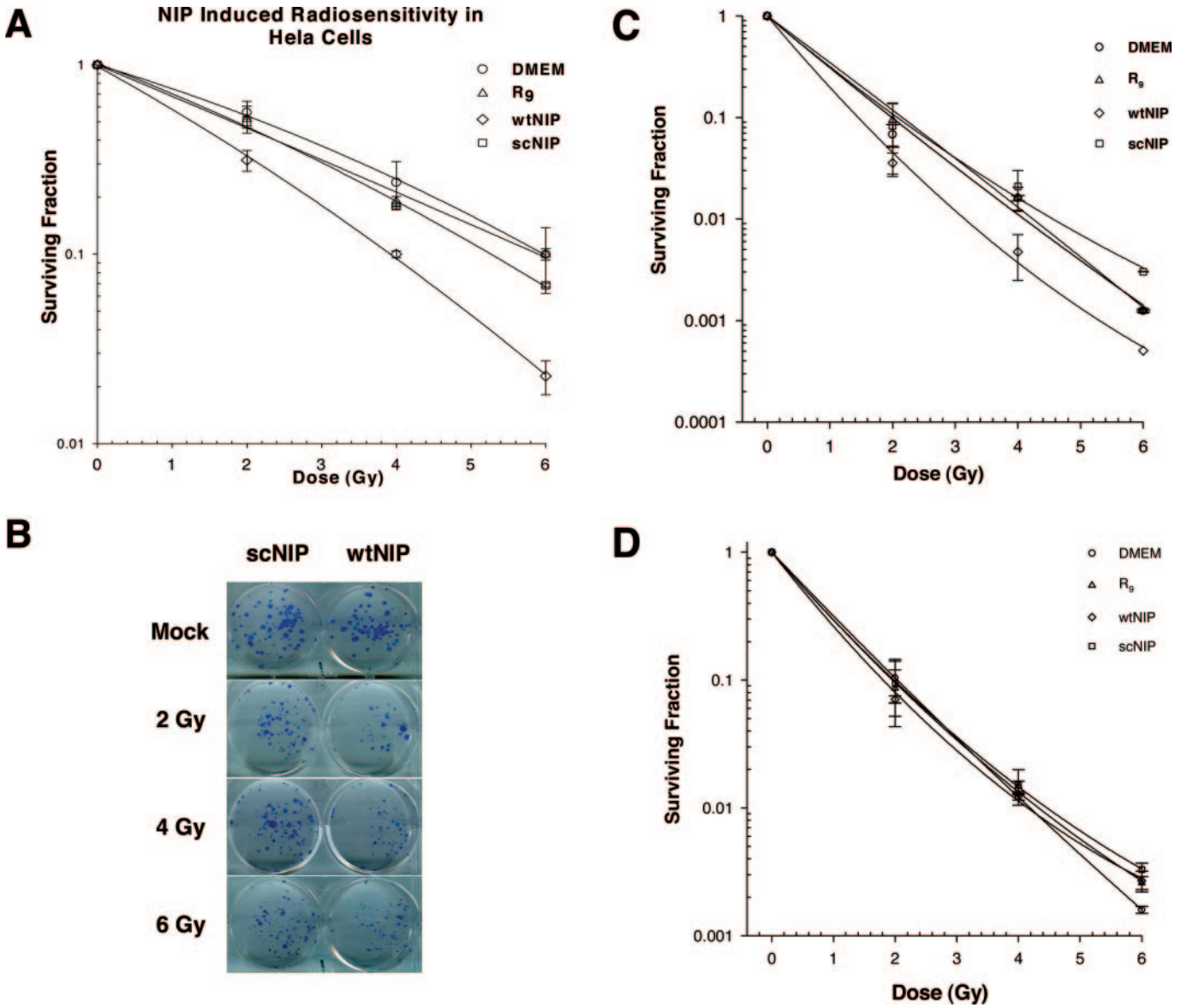

Fig. 6. WtNIP increases cellular radiosensitivity. Cells were seeded at limiting dilutions and treated with 10 μM R_9, wtNIP, or scNIP for 1 h before irradiation, continuously exposed to the peptides for 24 h, harvested 10 to 12 days later, and stained with crystal violet. Shown in A (HeLa), C (MO59J), and D (GM9607) are the surviving curves after indicated doses of radiation. Error bars represent ± 1 S.E.M.; graphed are the mean of three independent experiments. B, representative plates of the clonogenic assay for NIP-mediated radiosensitivity in HeLa cells.

inhibitors for further elucidation of signaling pathways involved in the DNA damage response. However, the use of the polyarginine-mediated NBS1 peptide as a therapeutic agent still faces challenges such as peptide stability, toxicity, tumor specific targeting, and immunogenic effects, etc. Using the current concept to establish an assay for high-throughput screening to identify small molecules that can target the NBS1-ATM interaction will eventually lead to novel radiosensitizers usable for clinical settings. Future studies are also necessary to determine the structure of the NBS1-ATM interaction complex and how wtNIP competes with the interaction.

Acknowledgments

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MOL PHARM

Correction to "Characterization of an NBS1 C-Terminal Peptide That Can Inhibit Ataxia Telangiectasia Mutated (ATM)-Mediated DNA Damage Responses and Enhance Radiosensitivity"

In the above article [Cariveau M, Tang X, Cui XL, and Xu B (2007) *Mol Pharmacol* **72:**320–326], Fig. 4 was incorrect (identical to Fig. 5), but the legend was correct. The correct Fig. 4 is shown below with the original correct legend.

The online version of this article have been corrected in departure from the print version.

The printer regrets this error and apologizes for any confusion or inconvenience it may have caused.

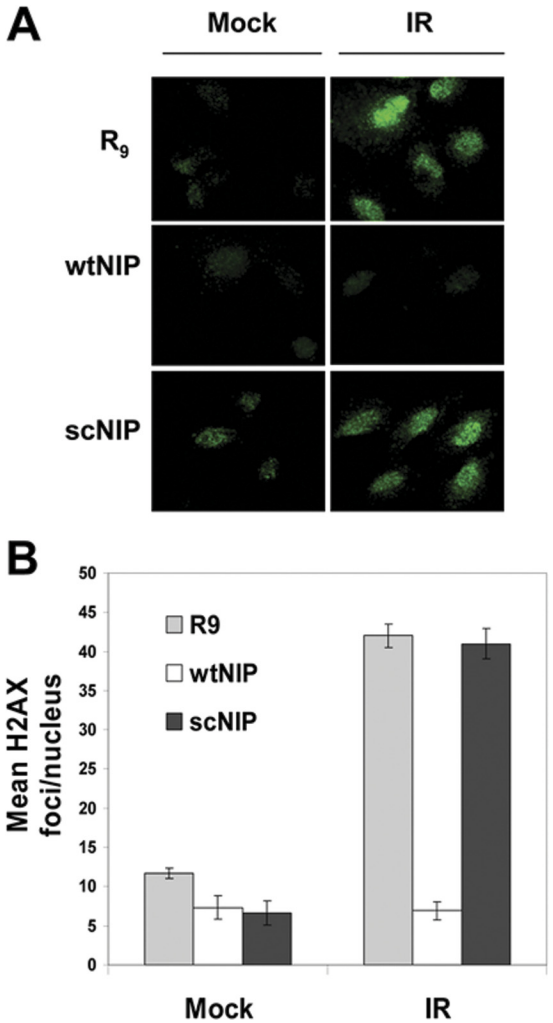

Fig. 4. WtNIP can inhibit γ-H2AX focus formation. A, HeLa cells were treated with 10 μM R_9, wtNIP, or scNIP for 1 h, irradiated with 0 or 6 Gy, and harvested 30 min later before immunofluorescence microscopy was employed to detect radiation-induced γ-H2AX foci. B, the mean γ-H2AX nuclear foci per nucleus were determined for each image using Image-Pro Plus 5.1 software and is expressed in arbitrary units. Error bars represent ± 1 S.D.; graphed are the mean of three independent experiments.