

Inhibition of Hsp90 Compromises the DNA Damage Response to Radiation

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Abstract

Inhibitors of the molecular chaperone Hsp90 have been shown to enhance tumor cell radiosensitivity. To begin to address the mechanism responsible, we have determined the effect of the Hsp90 inhibitor 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17DMAG) on the DNA damage response to radiation. Exposure of MiaPaCa tumor cells to 17DMAG, which results in radiosensitization, inhibited the repair of DNA double-strand breaks according to γ H2AX foci dispersal and the neutral comet assay. This repair inhibition was associated with reduced DNA-PK catalytic subunit (DNA-PKcs) phosphorylation after irradiation and a disruption of DNA-PKcs/ErbB1 interaction. These data suggest that the previously established 17DMAG-mediated reduction in ErbB1 activity reduces its interaction with DNA-PKcs and thus accounts for the attenuation of radiation-induced DNA-PK activation. 17DMAG was also found to abrogate the activation of the G₂- and S-phase cell cycle checkpoints. Associated with these events was a reduction in radiation-induced ataxia-telangiectasia mutated (ATM) activation and foci formation in 17DMAG-treated cells. Although no interaction between ATM and Hsp90 was detected, Hsp90 was found to interact with the MRE11/Rad50/NBS1 (MRN) complex. 17DMAG exposure reduced the ability of the MRN components to form nuclear foci after irradiation. Moreover, 17DMAG exposure reduced the interaction between NBS1 and ATM, although no degradation of the MRN complex was detected. These results suggest that the diminished radiation-induced activation of ATM in 17DMAG-treated cells was the result of a compromise in the function of the MRN complex. These data indicate that Hsp90 can contribute to the DNA damage response to radiation affecting both DNA repair and cell cycle checkpoint activation. (Cancer Res 2006; 66(18): 9211-20)

Introduction

Hsp90 is a highly expressed molecular chaperone that plays a critical role in the stabilization and regulation of a wide variety of proteins (1, 2). Because it is expressed at a higher level in malignant versus normal cells and because its client proteins assume critical roles in tumor initiation and progression, Hsp90 has received considerable attention as a potential target for cancer therapy. In addition, Hsp90 has been implicated as a determinant of tumor cell

radiosensitivity. Initial studies into the role of Hsp90 in radioresistance were predicated on its stabilization of signaling molecules that had already been associated with protection against radiation-induced cell death (3). Thus, whereas most efforts to develop target-based strategies for enhancing tumor radiosensitivity had focused on individual radioresponse-associated molecules, inhibition of Hsp90 provides an approach for the simultaneous targeting of multiple proteins involved in radioresponse. The putative advantages of such a multitarget strategy are increases in the degree and probability of radiosensitization. Indeed, inhibitors of Hsp90, such as geldanamycin, 17-allylamino-geldanamycin, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17DMAG), and radicicol, have been shown to significantly enhance the radiosensitivity of tumor cell lines derived from a variety of histologies (3–8). Moreover, for reasons yet to be defined, this radiosensitization was found to be selective for tumor cells over normal fibroblast cell lines (3, 5).

Although Hsp90 seems to be a clinically relevant target for enhancing tumor radioresponse, translating these experimental results into a treatment situation would benefit from a thorough understanding of the mechanisms involved. Toward this end, we have focused on the Hsp90 inhibitor 17DMAG, which is under clinical evaluation (2),⁵ 17DMAG was found to enhance the *in vitro* radiosensitivity of human cell lines originating from three different tumor histologies and to result in a greater than additive increase in radiation-induced tumor growth delay in a human tumor xenograft model (4). Whereas a number of radioresponse-associated proteins were degraded in response to 17DMAG exposure, the radiosensitization seemed to correlate best with a decrease in the levels of ErbB2 (4). In a subsequent study, however, we reported on cell lines in which 17DMAG induced the degradation of ErbB2, yet had no effect on radiosensitivity (9). The lack of an enhancement in radiosensitivity despite ErbB2 degradation was then shown to be the result of ErbB3-dependent stabilization of ErbB1 activity in the presence of 17DMAG-mediated ErbB2 degradation. This stabilization was only present in cells resistant to 17DMAG-induced radiosensitization. However, whereas this study showed that the loss of ErbB1 activity is necessary for 17DMAG-induced radiosensitization, in cell lines sensitized by 17DMAG, treatment with small interfering RNA to ErbB2, which reduced ErbB1 activity, had no effect on radiosensitivity. Thus, these results suggest that, whereas the loss of ErbB1 signaling is necessary for 17DMAG-induced radiosensitization, it is not sufficient, indicating the existence of additional critical targets.

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Investigations into the mechanism through which Hsp90 regulates tumor cell radiosensitivity have focused primarily on the role of cytoplasmic signaling molecules; the cellular processes that mediate radiation-induced cell death, however, have not been addressed in detail. Cellular radiosensitivity is determined by a number of fundamental processes, such as apoptosis, cell cycle phase distribution, cell cycle checkpoint activation, and the repair of DNA damage, specifically double-strand breaks (DSB). Delineating which of these processes participates in 17DMAG-induced radiosensitization should not only provide mechanistic insight, but provide a framework for investigations into the specific molecules involved, i.e., the critical targets. We had previously reported that the radiosensitization induced by Hsp90 inhibition does not involve synchronization of cells into a radiosensitive phase of the cell cycle or an increase in susceptibility to apoptotic death (3, 4). These studies have now been expanded to an evaluation of DNA repair and cell cycle checkpoint activation, critical components of the DNA damage response. The data presented indicate that 17DMAG inhibits the repair of radiation-induced DSBs and abrogates radiation-induced activation of cell cycle checkpoints. Moreover, these defects are associated with reductions in the radiation-induced activation of DNA-PKcs and ataxia-telangiectasia mutated (ATM) in 17DMAG-treated cells.

Materials and Methods

Cell lines and treatment. The MiaPaCa and ASPC1 (human pancreatic carcinoma) cell lines were obtained from American Type Culture Collection (Gaithersburg, MD) and grown in as described (9). 17DMAG was provided by the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program of the National Cancer Institute, and dissolved in PBS. Cultures were irradiated using a Pantak (Solon, OH) X-ray source at a dose rate of 1.55 Gy/min.

Immunofluorescence. Immunofluorescence was done as previously described (10). Briefly, cells grown in tissue culture slides were fixed with 4% paraformaldehyde, permeabilized with 0.2% NP40, and blocked with 1% bovine serum albumin (BSA) in PBS containing 5% goat serum. For NBS1 staining, cells were fixed with 100% methanol at -20°C , rinsed with ice-cold acetone, air-dried, and blocked with 10% BSA. The slides were incubated with primary antibodies overnight at 4°C and with secondary antibodies (Alexa Fluor 488 goat anti-mouse or goat anti-rabbit IgG and Alexa Fluor 594 goat anti-rabbit or goat anti-mouse IgG at 1:500, Molecular Probes, Eugene, OR) and 4',6-diamidino-2-phenylindole in PBS with 1% BSA. Primary antibodies used for immunostaining were phospho-H2AX (Upstate Biotechnology, Charlottesville, VA), phospho-ATM (Rockland Immunochemicals, Gilbertsville, PA), MRE11 (Genetex, San Antonio, TX), RAD50 (Genetex), and NBS1 (Novus Biologicals, Inc., Littleton, CO). Images were captured using a Leica DMRXA fluorescent microscope (Leica, Wetzlar, Germany) and a Photometrics Sensys charge coupled device camera (Roper Scientific, Tucson, AZ) and imported into IP Labs software (Signal Analytics Corporation, Vienna, VA).

Neutral comet assay. As a measure of DNA DSBs, the neutral comet assay was done using a kit from Trevigen according to the recommendations from the manufacturer (Trevigen, Gaithersburg, MD). DNA was stained by propidium iodide and digital fluorescent images were obtained using the IP Labs software (Signal Analytics Corporation). At least 50 images per point were evaluated with Olive Tail Moment determined using CASP software (11).

G₂ arrest. To evaluate the activation of the G₂ cell cycle checkpoint, mitotic cells were distinguished from G₂ cells and the mitotic index was determined according to the expression of phosphorylated histone H3 (Upstate Biotechnology) as detected in the 4N population using the flow cytometric method of Xu et al. (12).

Inhibition of DNA synthesis (S-phase arrest). The analysis of DNA synthesis, based on the incorporation of BrdUrd into the genomic DNA, was

done using the Cell Proliferation ELISA kit (Roche Applied Science, Indianapolis, IN). Immediately after irradiation, growth medium was replaced with 17DMAG-free medium containing BrdUrd (10 $\mu\text{mol/L}$). One hour later, the cells were fixed and the amount of BrdUrd incorporated into DNA determined.

Preparation of whole cell extracts and subcellular fractionation. Cell extracts were prepared as described previously (9). For extraction of the cytosolic and nuclear protein, cells were lysed in ice-cold hypotonic lysis buffer [10 mmol/L HEPES (pH 7.9), 60 mmol/L KCl, 1 mmol/L EDTA, 0.3% NP40, 1 mmol/L NaF, 1 mmol/L NaO₃V₄, and protease inhibitor cocktail (Roche Diagnostic Corp., Indianapolis, IN)] with occasional vortexing for 30 minutes. The lysate was centrifuged at 1,600 rpm for 5 minutes to pellet nuclei; the supernatant was collected as cytoplasmic protein. Nuclei were washed with ice-cold hypotonic buffer without NP40, resuspended in nuclear extraction buffer [20 mmol/L Tris-HCl (pH 7.5), 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 25% glycerol, 1 mmol/L NaF, 1 mmol/L NaO₃V₄, and protease inhibitor cocktail], vortexed at 4°C for 30 minutes, centrifuged at 15,000 rpm for 5 minutes, and the supernatant was collected as nuclear extracts. Protein concentrations were measured using a modification of the Bradford method or a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) according to the instructions from the manufacturer.

Immunoprecipitation and immunoblot analysis. For immunoblots, lysates (20 μg) were electrophoresed on SDS-polyacrylamide gels. For immunoprecipitation, 500 μg of whole cell lysate or 300 μg of nuclear protein were incubated in binding buffer 1 [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, and protease inhibitor cocktail] or binding buffer 2 [10 mmol/L HEPES (pH 7.9), 100 mmol/L NaCl, 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 250 mmol/L sucrose, and protease inhibitor cocktail], respectively, with the indicated primary antibody. The next day, 40 μL protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the lysate and incubated for 1.5 hours at 4°C . Immunoprecipitates were washed thrice with cold binding buffer and resuspended in sample buffer, heated at 90°C for 10 minutes, and centrifuged. Supernatants were electrophoretically transferred at 100 V for 2 hours to Immobion-P membranes. Membranes were blocked with 5% nonfat dry milk in 500 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), and 0.1% Tween 20 (TBST) for 1 hour followed by incubation with primary antibody, washing in TBST, and incubation with horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution in 3% nonfat dry milk/TBST; Santa Cruz Biotechnology). Blots were developed using Western blotting Luminol reagent (Santa Cruz Biotechnology) or Visualizer Western blot detection kit (Upstate, Lake Placid, NY) and the Luminescent Image Analyzer LAS-3000 (Fujifilm, Stamford, CT) according to instructions from the manufacturer. Antibodies against ATM (Western blot, Upstate; immunoprecipitation, Genetex), phospho-ATM (Western blot, Rockland Immunochemicals), DNA-PKcs (Western blot; Lab Vision Corp., Fremont, CA), phospho-DNA-PKcs (Western blot, Abcam, Cambridge, MA), Ku70 (Santa Cruz Biotechnology), Ku86 (Santa Cruz Biotechnology), ErbB1 (immunoprecipitation, Santa Cruz Biotechnology; Western blot, BD Biosciences, San Diego, CA), Mre11 (Western blot, Genetex; immunoprecipitation, EMD Biosciences, Inc., San Diego, CA), Rad50 (Western blot and immunoprecipitation, Upstate), NBS1 (Western blot and immunoprecipitation, Genetex); Hsp90 (Western blot, Santa Cruz Biotechnology; immunoprecipitation, Santa Cruz Biotechnology), calnexin (Western blot, BD Biosciences); lamin B and actin (Chemicon, Temecula, CA) were purchased from commercial sources.

Results

To generate insight into the mechanism through which Hsp90 influences radiosensitivity, the effects of the Hsp90 inhibitor 17DMAG were determined on components of the DNA damage response in the MiaPaca pancreatic carcinoma cell line. We previously reported that exposure to 17DMAG (50 nmol/L) for 16 hours enhances the radiosensitivity of MiaPaca cells with a dose enhancement factor of 1.6 (9). With respect to cell death, the

critical DNA lesion induced by ionizing radiation is the DSB. To determine the effects of 17DMAG on this form of DNA damage, we evaluated foci of phosphorylated histone H2AX (γ H2AX), which has been established as a sensitive indicator of DSBs (13–15). Cells were exposed to 17DMAG, irradiated (2 Gy), fed fresh 17DMAG-free medium, and the number γ H2AX foci was determined (Fig. 1A). The 16-hour exposure to 17DMAG alone had no effect on the number of γ H2AX foci, suggesting that Hsp90 inhibition does not induce DNA damage, at least not DSBs. The 2 Gy dose alone induced an increase in the number of γ H2AX foci at 1 hour, which was reduced by 6 hours and returned to background levels by 24 hours after irradiation, consistent with previous reports (15). For cells exposed to the combination of 17DMAG and radiation, there was no difference in the level of γ H2AX foci compared with radiation only at the 1-hour time point, suggesting that Hsp90 inhibition does not influence the initial level of DSB induction. However, at the 6 and 24 hours, the number of γ H2AX foci in

cultures receiving the combined 17DMAG/radiation treatment was significantly greater compared with the radiation only group. The delayed dispersal of γ H2AX foci suggests that 17DMAG inhibits the repair of DSBs induced by radiation (15, 16).

Whereas γ H2AX is considered an indicator of radiation-induced DSBs and its expression at 24 hours after irradiation correlates with radiosensitivity (16), it reflects a chromatin level response. Therefore, as an additional measure of the effects of 17DMAG on radiation-induced DSBs, the neutral comet assay was done (17). MiaPaca cells were exposed to 17DMAG for 16 hours before irradiation; cells were collected for the neutral comet assay immediately after irradiation and at times out to 6 hours. As shown in Fig. 1B, in cells exposed to 17DMAG before irradiation, there was a significant increase in the percentage of DNA damage remaining compared with radiation alone. Exposure of MiaPaca cells to 17DMAG alone had no effect on DNA damage, nor did 17DMAG affect the initial level of DNA damage induced by radiation (data not shown). Thus, consistent with the γ H2AX data, these results indicate that 17DMAG inhibits the repair of radiation-induced DSBs.

To address the specific molecules involved in the 17DMAG-mediated inhibition of DSB repair, we focused on DNA-PK, which is a critical component of the nonhomologous end joining process and has recently been associated with ErbB1 activity (18). DNA-PK is composed of a catalytic subunit (DNA-PKcs) and the regulatory Ku subunits (19). The activation of DNA-PKcs involves autophosphorylation at Thr-2609 (20). To determine whether 17DMAG affects radiation-induced activation of DNA-PKcs, the Thr-2609 phosphorylation status of DNA-PKcs was determined (Fig. 2A). DNA-PKcs phosphorylation was not detectable in control cells or in those exposed to 17DMAG only. Radiation (4 Gy) alone induced a rapid DNA-PKcs phosphorylation consistent with the previous published results (20). However, in 17DMAG-treated cells, the radiation-induced phosphorylation of DNA-PKcs was attenuated. The levels of total DNA-PKcs, Ku70 and Ku86, were not affected by radiation and/or 17DMAG. Thus, these data suggest that whereas 17DMAG inhibits radiation-induced activation of DNA-PKcs, the mechanism does not involve a reduction in one of the components of the DNA-PK complex. As shown in the bottom panel, 17DMAG exposure had no effect on the radiation-induced activation of DNA-PKcs in ASPC1 cells, which are resistant to 17DMAG-induced radiosensitization (9). It should be noted that 17DMAG also had no effect on the repair of radiation-induced DSBs in ASPC1 cells (data not shown).

Recently, it has been reported that DNA-PK activity can be regulated by nuclear ErbB1 (18). Previously, we showed that exposure of MiaPaca cells to 17DMAG decreases ErbB1 phosphorylation and activity (9), which is apparently necessary for its nuclear transport and subsequent interaction with DNA-PK (18). Therefore, to address the possible relationship between the 17DMAG-mediated reductions in ErbB1 activity and radiation-induced DNA-PKcs activation, the effects of 17DMAG on the radiation-induced ErbB1/DNA-PK interaction were determined. MiaPaca cells were exposed to 17DMAG followed by irradiation, and nuclei were collected 20 minutes later and subjected to coimmunoprecipitation of ErbB1 and DNA-PKcs. As shown in Fig. 2B, radiation alone induced the interaction of ErbB1 and DNA-PKcs, consistent with the previous results (18). However, in 17DMAG-treated cells, the radiation-induced ErbB1/DNA-PK interaction was reduced. These results suggest that the 17DMAG-mediated inhibition of radiation-induced DSB repair involves a

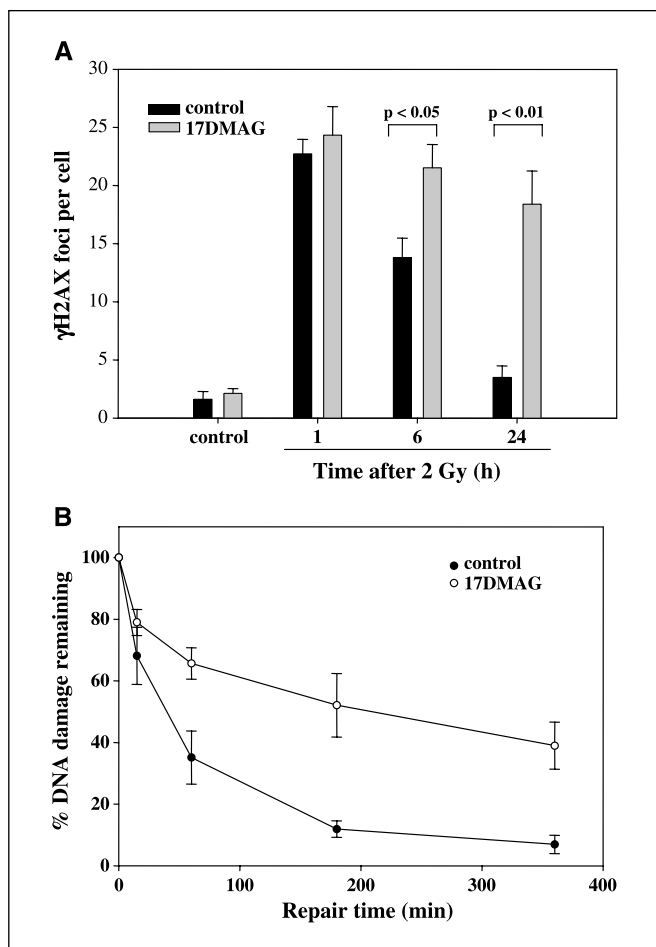


Figure 1. Effects of 17DMAG on the repair of radiation-induced DSBs. *A*, radiation-induced γ H2AX foci. MiaPaca cells grown in chamber slides were exposed to 17DMAG (50 nmol/L) for 16 hours, irradiated (2 Gy), and fixed at the specific time for analysis of nuclear γ H2AX foci. *Black columns*, vehicle-treated cells; *gray columns*, cells that had been exposed to 17DMAG. Foci were evaluated in 100 nuclei per treatment. *Columns*, mean; *bars*, SD. *B*, analysis DSB repair using neutral comet assay. MiaPaca cells were exposed to 17DMAG (50 nmol/L) for 16 hours, irradiated (10 Gy), and collected for analysis at the specified times. For the determination of initial induction of DNA damage (0 minute), cells were irradiated on ice. Data are presented as percentage of initial radiation-induced damage remaining. *Points*, mean from three independent experiments; *bars*, SD.

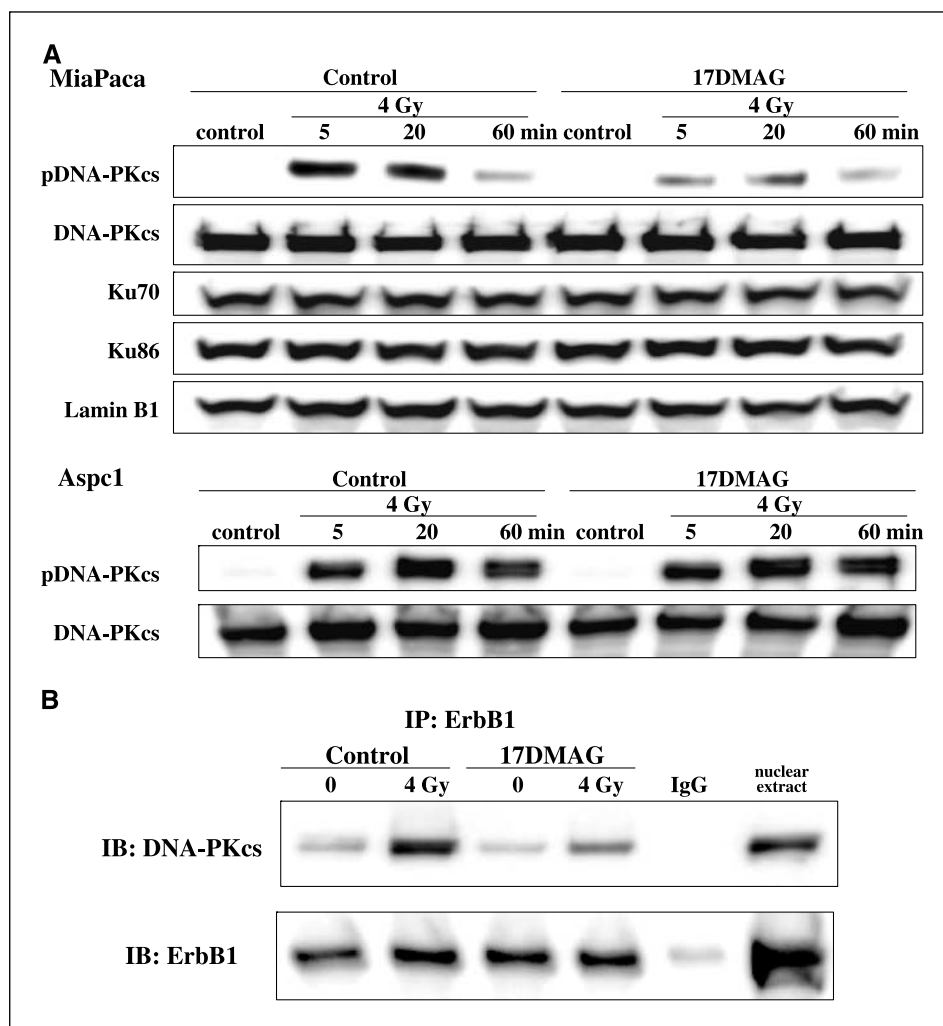


Figure 2. Effects of 17DMAG on radiation-induced DNA-PK. Cells were exposed to 17DMAG (50 nmol/L) for 16 hours, irradiated (4 Gy), and harvested at the specified times. *A*, nuclear extracts (20 μ g) were subjected to immunoblot analysis of the DNA-PK components after exposure to 17DMAG and/or 4 Gy. Representative of two independent experiments for each cell line using lamin B1 as a loading control. *B*, interaction between DNA-PKcs and ErbB1 after treatment with 17DMAG and/or 4 Gy. Nuclear extracts (150 μ g) were collected at 20 minutes after irradiation and immunoprecipitated (*IP*) with the ErbB1 antibody. Immunoprecipitates were analyzed by immunoblotting (*IB*) for ErbB1 and DNA-PKcs. Each blot is representative of two independent experiments.

reduction in the interaction between ErbB1 and DNA-PKcs and consequently DNA-PK activity.

In addition to repair processes, the DNA damage response includes the activation of cell cycle checkpoints. We had previously reported that exposure of DU145 cells to 17DMAG resulted in an abrogation of radiation-induced activation of G₂- and S-phase arrest (4). To more completely investigate this effect and address the specific molecules involved, these studies were extended to MiaPaca cells. The effects of 17DMAG on radiation-induced G₂ arrest were evaluated using the method of Xu et al. (12), which, based on the expression of phosphorylated histone H3, distinguishes between G₂ and mitotic cells, providing a measure of the mitotic index. Irradiation of MiaPaCa cells resulted in a rapid reduction in mitotic cells (Fig. 3A), consistent with the rapid onset of G₂ arrest and with previously published results (12). Exposure to 17DMAG alone, which was rinsed off, and feeding cells with fresh growth medium immediately after irradiation (0 time point), resulted in an increase in the percentage mitotic cells out to at least 6 hours. The mitotic index for cells exposed to the 17DMAG/radiation combination did not decrease as for radiation alone, but increased in a manner similar to cells exposed to 17DMAG alone, indicating that 17DMAG abrogates radiation-induced G₂ arrest. To address the effects of 17DMAG on the S-phase checkpoint, radioresistant DNA synthesis (21) was evaluated after irradiation

(Fig. 3B). Radiation alone resulted in a decrease in DNA synthesis corresponding to the activation of the S-phase checkpoint. However, in cells exposed to 17DMAG for 16 hours before irradiation, DNA synthesis was essentially unchanged compared with unirradiated cells. These data indicate that in addition to the abrogation of G₂ arrest, 17DMAG inhibits radiation-induced S-phase arrest, consistent with previous studies using DU145 cells (4).

A molecule that plays a critical role in regulating both G₂- and S-phase arrest after irradiation is ATM (22). Therefore, we hypothesized that 17DMAG inhibits radiation-induced ATM activation, which involves the autophosphorylation of Ser-1981 (23). As shown in Fig. 4A, ATM was phosphorylated within 5 minutes after irradiation of MiaPaca cells, consistent with previous results (23). However, in MiaPaca cells exposed to 17DMAG, the level of radiation-induced ATM phosphorylation was reduced at each of the time points evaluated. As shown in the bottom panel, 17DMAG exposure had no effect on the radiation-induced activation of ATM in ASPC1 cells, which are resistant to 17DMAG-induced radiosensitization (9). It should be noted that 17DMAG also had no effect on radiation-induced G₂ arrest in ASPC1 cells (data not shown). To further explore the influence of 17DMAG on radiation-induced ATM activation, the induction of phospho-ATM foci, which form at the site of DSBs (23), was

evaluated in MiaPaca cells (Fig. 4B). In control cells, as in previous reports (23), radiation induced the formation of phospho-ATM foci within 5 minutes. In comparison, the number of radiation-induced phospho-ATM foci in 17DMAG-treated cells was clearly reduced. By 180 minutes, whereas essentially all the cells in the irradiation alone control group contained >10 foci, in those cells exposed to 17DMAG >30% of cells contained <10 foci (Fig. 4C). Thus, the results presented in Fig. 4 indicate that 17DMAG inhibits the radiation-induced activation of ATM.

Recent reports indicate that the MRN complex, which is composed of MRE11, RAD50, and NBS1, is required for the optimal activation of ATM (24–26). Therefore, to further investigate the relationship between Hsp90 and ATM activation, the components of the MRN complex were evaluated in MiaPaca cells exposed to

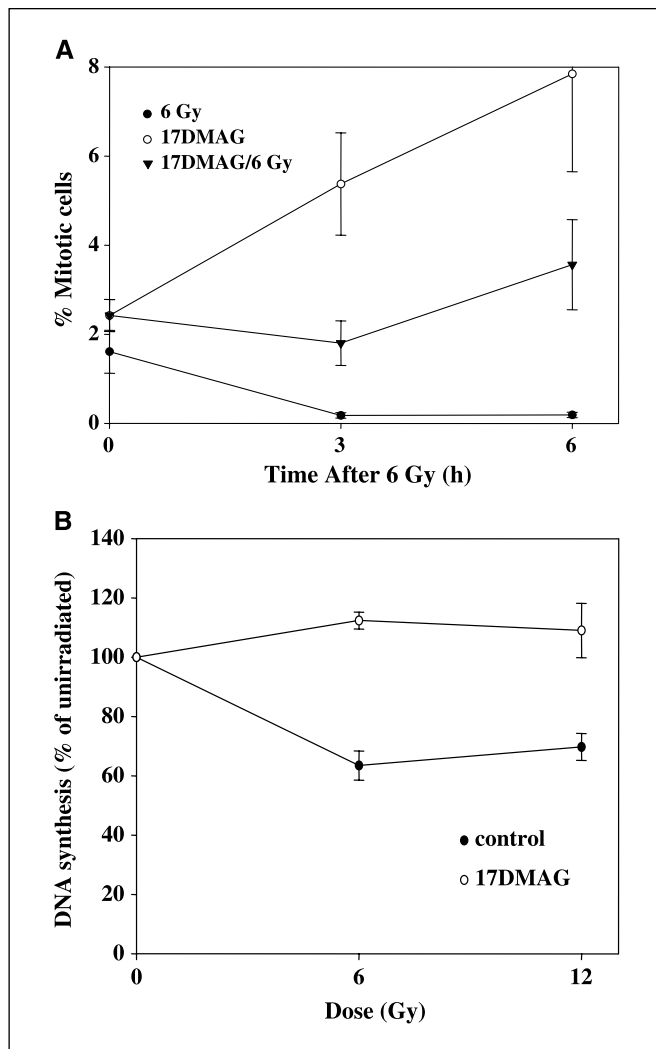


Figure 3. Abrogation of radiation-induced cell cycle checkpoints by 17DMAG. *A*, radiation-induced G₂ arrest. MiaPaca cells were exposed to 17DMAG (50 nmol/L) for 16 hours, irradiated (6 Gy), then fed 17DMAG-free medium and collected at the specified times for determination of mitotic index (percent mitotic cells). Points, mean of four independent experiments; bars, SE. The differences between the 6 Gy and the 17-DMAG/6 Gy values were significant at each time point ($P < 0.03$, 6 Gy versus 17-DMAG/6 Gy). *B*, radiation-induced, S-phase arrest. MiaPaCa cells were treated with 17DMAG as above, irradiated, and 17DMAG-free medium containing BrdUrd (10 μ mol/L) was added; cells were then collected 1 hour later. Points, mean of three independent experiments; bars, SE. The difference between the radiation and 17DMAG + radiation values were significant at each dose ($P < 0.03$ at 6 Gy and $P < 0.02$ at 12 Gy).

the combination of 17DMAG and radiation. As shown in Fig. 5A, the expression of the individual MRN components in MiaPaca cells was unchanged after exposure to radiation and/or 17DMAG. In addition, based on coimmunoprecipitation experiments, 17DMAG did not dramatically affect MRN complex composition (Fig. 5B). Although 17DMAG reproducibly reduced the amount of NBS1 coimmunoprecipitating with MRE11 and RAD50, this effect was not detected in the reciprocal experiment in which NBS1 was immunoprecipitated. After irradiation, MRE11, RAD50, and NBS1 form nuclear foci, a process that contributes to their function in the DNA damage response (27, 28). Thus, as an evaluation of MRN function, immunocytochemistry was used to visualize foci formation (29). Irradiation of MiaPaca cells resulted in the formation of distinct foci containing MRE11, RAD50, and NBS1 (Fig. 5C), consistent with previous reports (27, 28). However, in irradiated 17DMAG-treated cells, although each protein was present, there seemed to be a reduction in the ability of MRE11, RAD50, and NBS1 to coalesce into foci. These micrographs suggest that the 17DMAG-mediated inhibition of Hsp90 influences MRN function.

To determine whether Hsp90 directly associates with the MRN complex, Hsp90 was immunoprecipitated from nuclear extracts and probed for the MRN components as well as ATM (Fig. 6). Immunoprecipitation with anti-Hsp90 antibody resulted in the coprecipitation of each of the MRN components. ATM did not coprecipitate with Hsp90. Given that RAD50, NBS1, and MRE11 exist in a stable complex, it is not possible at this time to determine whether Hsp90 interacts with each of the individual components of MRN or with just one of the components, and is simply pulled down with the complex. However, given the significance of NBS1 in directing the MRN complex to sites of DNA damage and in the activation of ATM (26, 30), the reciprocal experiment was done. That is, NBS1 was immunoprecipitated from MiaPaca nuclear extracts and probed for Hsp90. Coprecipitating with NBS1 was Hsp90 along with Rad50 and MRE11. These data indicate that MRN or a protein in the complex binds to Hsp90.

NBS1 has been shown to weakly bind to inactive ATM and in response to DNA damage to participate in ATM activation as well as its recruitment to the site of DSBs (26, 30). Therefore, to pursue the potential functional consequences of the Hsp90/NBS1 interaction, we determined whether 17DMAG affected the binding of NBS1 to ATM. Under control conditions, both Hsp90 and ATM were detected in NBS1 immunoprecipitates (Fig. 6B). In extracts isolated from 17DMAG-treated cells, however, the amount of Hsp90 and ATM coprecipitating with NBS1 was substantially reduced. The bottom panel shows the reciprocal experiment in which ATM was immunoprecipitated from MiaPaca nuclear extracts. Similar results were obtained in that NBS1 was found to coprecipitate with ATM in control cells, with its levels substantially reduced in 17DMAG-treated cells. These results suggest that nuclear Hsp90 physically associates with NBS1 (or another component of the MRN complex) and facilitates the NBS1/ATM interaction.

Discussion

Previous studies have shown that inhibition of Hsp90 enhances the radiosensitivity of cell lines initiated from a variety of human tumors, suggesting that this molecular chaperone is a clinically relevant target for tumor radiosensitization (3–8). On the other hand, it has also been shown that there are tumor cell lines resistant to the radiosensitizing actions of Hsp90 inhibition (9).

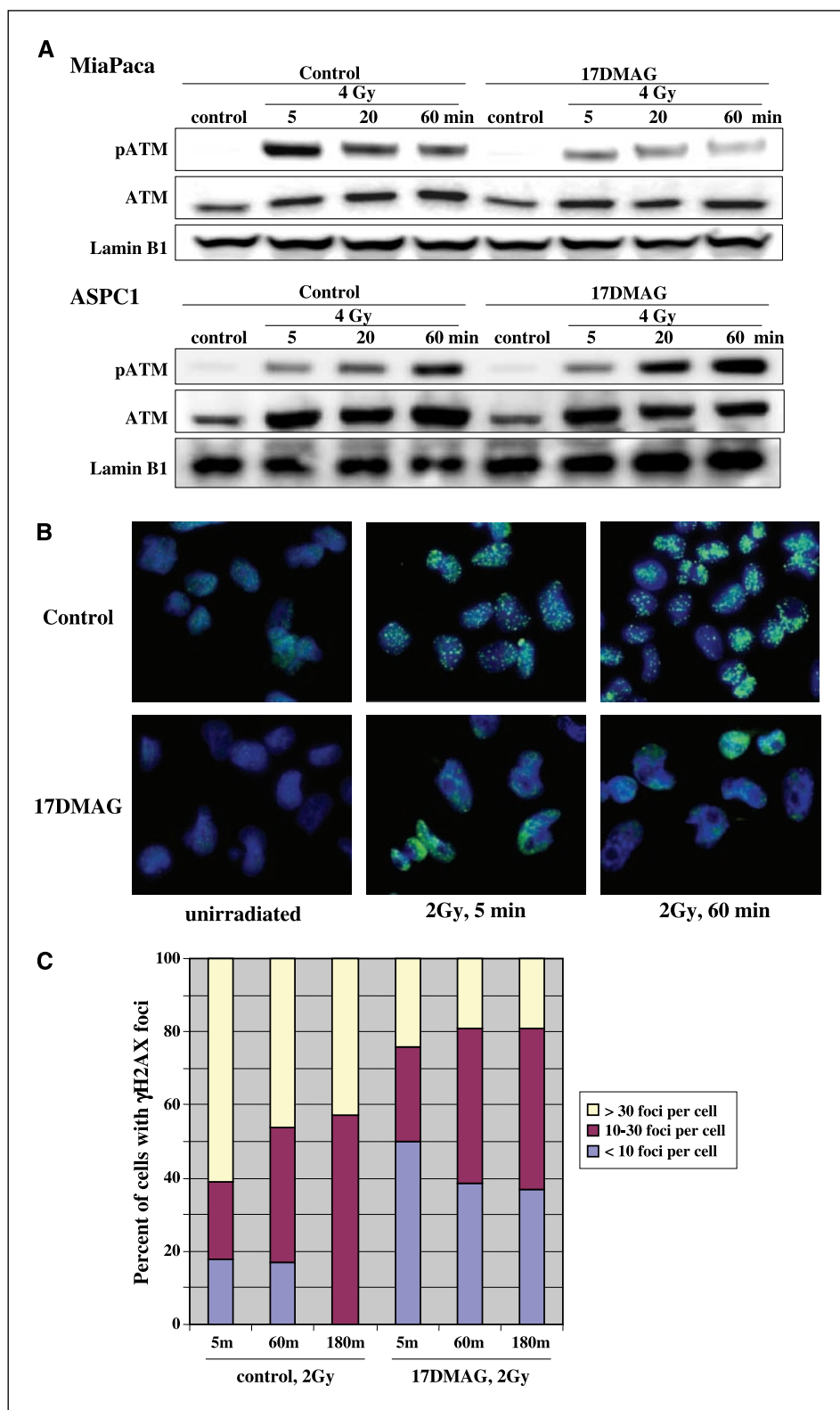


Figure 4. Effect of 17DMAG on radiation-induced ATM activation. *A*, radiation-induced ATM phosphorylation. Cells were exposed to 17DMAG (50 nmol/L) for 16 hours, irradiated (4 Gy), then fed 17DMAG-free medium; nuclear extracts were then collected at the specified times. Nuclear extracts were prepared and analyzed by sequential immunoblotting using a phosphospecific antibody to Ser-1981 of ATM, a panspecific antibody to ATM, and a monoclonal antibody to lamin B. Each blot is representative of two independent experiments. *B*, radiation-induced phospho-ATM foci formation in MiaPaCa cell. MiaPaCa cells grown in chamber slides were exposed to 17DMAG (50 nmol/L) for 16 hours, irradiated (2Gy), and fixed at the specific time for immunocytochemical analysis of nuclear phospho-ATM foci. Micrographs obtained from control cells and 17DMAG treated cells. *C*, quantification of phospho-ATM foci in irradiated cells with and without prior 17DMAG exposure. At least 100 cells were counted in each group and the data shown are representative of two independent experiments.

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Thus, as for other molecularly targeted agents, delineation of the mechanisms through which Hsp90 inhibitors affect radiosensitivity would not only be of fundamental interest, but may aid in defining the characteristics of the susceptible tumor cell phenotype. However, the putative advantage of targeting Hsp90, i.e., the loss

of multiple radioresponse-associated proteins, also severely complicates attempts to define the mechanisms involved. As an approach aimed at ultimately leading to the identification of the molecules that mediate the influence of Hsp90 on radioresistance, we have focused on defining the DNA damage response in

17DMAG-treated cells. The survival of cells after irradiation is heavily dependent on the DNA damage response, which consists of a series of integrated signaling pathways that participate in DNA repair and cell cycle checkpoint activation (31). Moreover, considerable information has been generated regarding the proteins involved as well as their interactions (32).

To determine the effects of 17DMAG-mediated Hsp90 inhibition on DSB repair, two methods were used— γ H2AX and neutral comet.

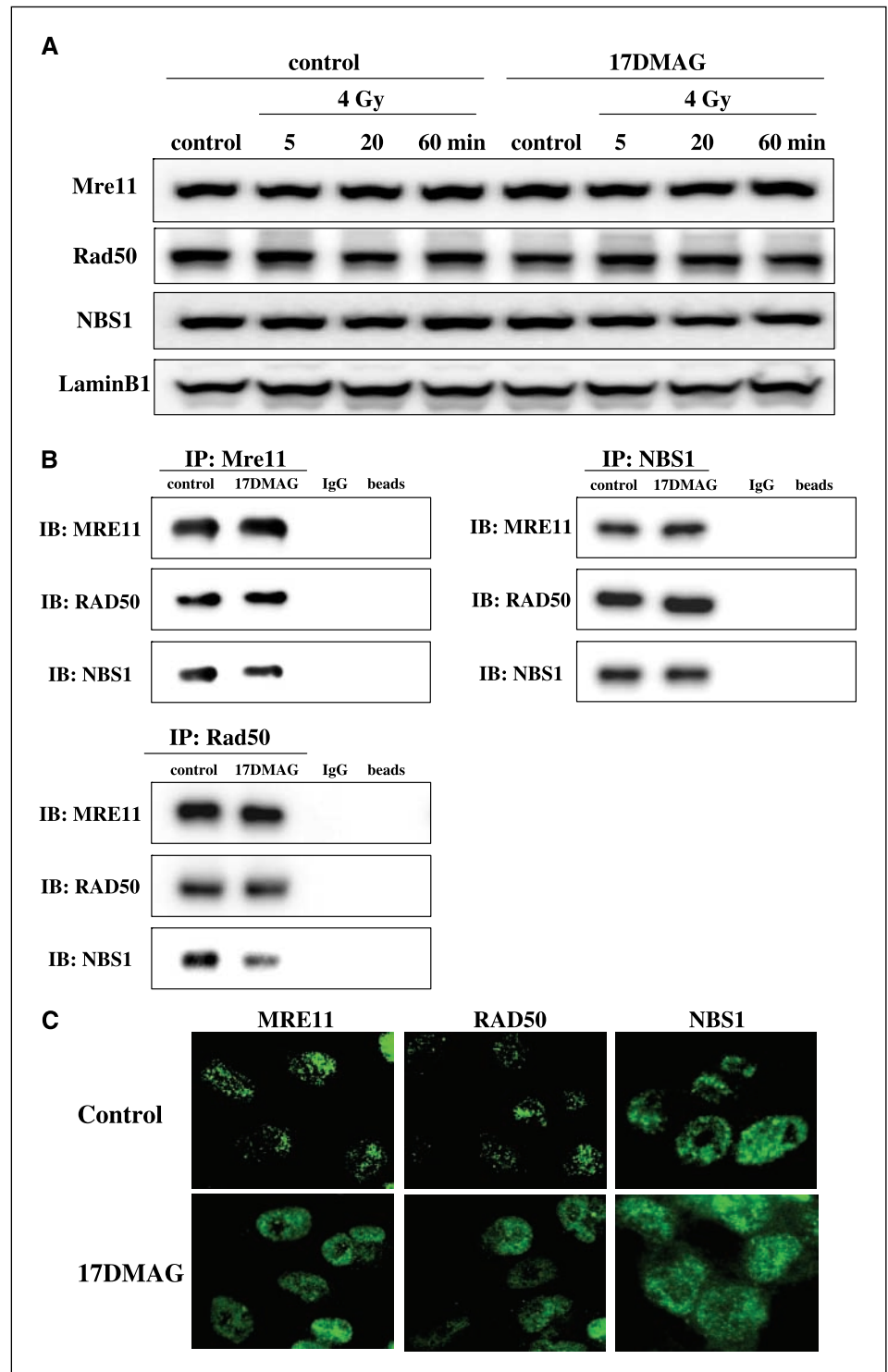
At sites of radiation-induced DSBs, the histone H2AX is rapidly phosphorylated (γ H2AX), forming readily visible nuclear foci (13, 14). Although the specific relationship between the dispersal of γ H2AX foci and the repair of a DSB has not been completely defined, the reduction in the number of γ H2AX foci in irradiated cells correlates with the repair of DSBs and cellular radiosensitivity (33, 34). An advantage to using γ H2AX foci as an indicator of radiation-induced DSBs is that they are readily detected at

Figure 5. Effects of 17DMAG on components of the MRN complex.

A. MiaPaCa cells were exposed to 17DMAG (50 nmol/L) for 16 hours, irradiated (4 Gy), and harvested at times out to 60 minutes. Nuclear extracts (20 μ g) from each time point were subjected to immunoblot analysis for Mre11, Rad50, and NBS1. Representative of two independent experiments for each cell line using lamin B1 as a loading control.

B. Influence of 17DMAG on the interaction of the MRN components. MiaPaCa cells were exposed to 17DMAG (50 nmol/L) for 16 hours. Cells were then collected and subjected to immunoprecipitation using antibodies to MRE11, RAD50, or NBS1 followed by immunoblot analyses for each of the other MRN components. IgG refers to extracts exposed to beads coated with nonspecific IgG and beads refers to extracts exposed to unlabeled beads. Representative of two independent experiments for each cell line using lamin B1 as a loading control.

C. Effects of 17DMAG on radiation-induced MRN foci formation. MiaPaCa cells were exposed to 17DMAG (50 nmol/L) for 16 hours, irradiated (2 Gy), and 1 hour later subjected to immunohistochemical analyses for the individual MRN components.



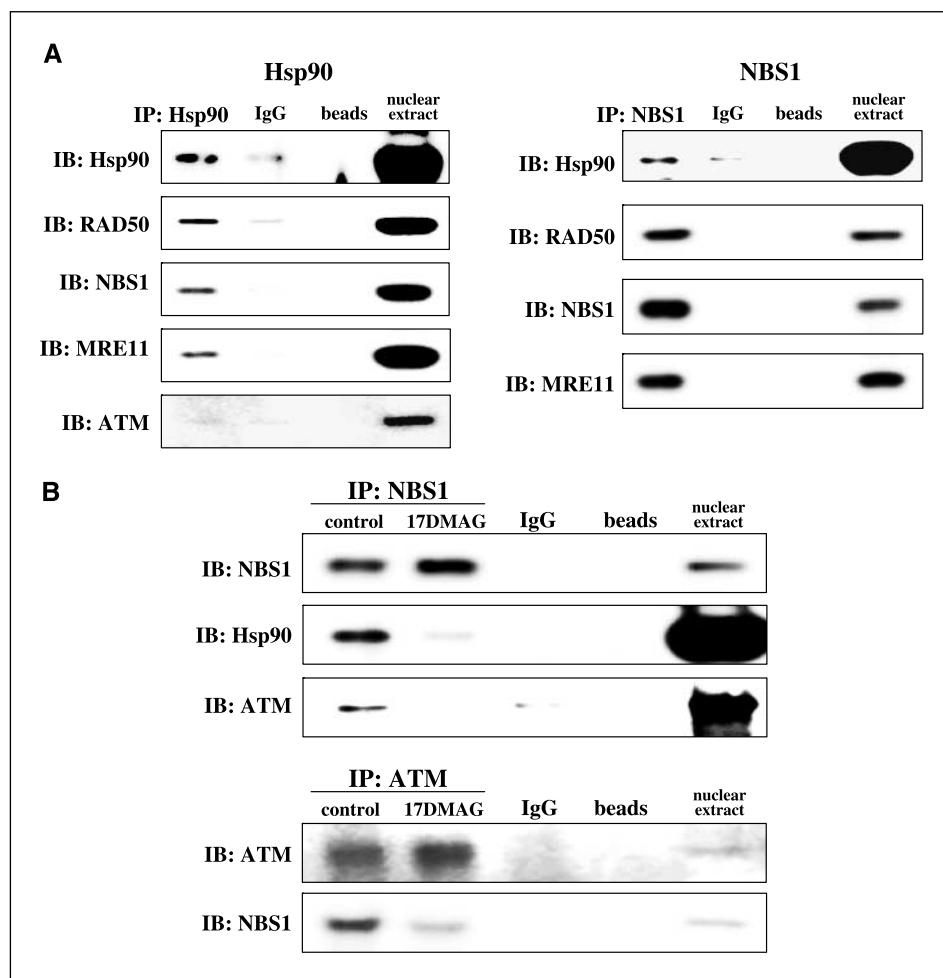


Figure 6. Interaction between nuclear Hsp90 and the components of the MRN complex. *A*, coimmunoprecipitation of Hsp90 and MRN complex. Nuclear extracts from MiaPaCa cells were subjected to immunoprecipitation using antibodies to Hsp90 or NBS1. IgG refers to extracts exposed to beads labeled with nonspecific IgG and beads refers to extracts exposed to unlabeled beads. Proteins from the immunoprecipitates were then analyzed by immunoblotting with 20 μ g of the nuclear extract used as a positive control. Each blot is representative of two independent experiments. *B*, effect of 17DMAG on the interaction between NBS1 and ATM. MiaPaCa cells were exposed to 17DMAG (50 nmol/L) for 16 hours followed by isolation of nuclear extracts, which were subjected to immunoprecipitation using antibodies to NBS1 or ATM or nonspecific IgG-coated agarose beads (IgG). Twenty micrograms of the nuclear extract were used as a positive control. Each blot is representative of two independent experiments.

clinically relevant doses (2 Gy). The comet assay provides a more direct measure of DNA strand breaks, selectively detecting DSBs over single-strand breaks when done under neutral conditions using 10 Gy (17). Both of these assays, which quantify different manifestations of DNA damage, indicate that 17DMAG inhibits the repair of radiation-induced DSBs. Thus, these initial results establish a role for Hsp90 in DNA repair.

Toward identifying the specific protein(s) mediating the 17DMAG-induced inhibition of DSB repair, we initially focused on DNA-PK. Falson et al. (35) reported that DNA-PKcs is a client of Hsp90 in HeLa cells, but not in normal HEK293 cells. Moreover, they reported that cytosolic but not nuclear DNA-PKcs levels were reduced in HeLa cells after treatment with an Hsp90 inhibitor (35). Treatment of MiaPaca cells with 17DMAG did not result in a loss of DNA-PKcs in the cytoplasm (data not shown) or the nucleus (Fig. 2), which may reflect the cell type specificity of this response. However, recent studies have also reported that the radiation-induced activation of nuclear DNA-PK can involve an interaction with ErbB1, which can be transported into nucleus after irradiation (18). We have recently shown that 17DMAG-induced radiosensitization involves the reduction in ErbB1 activity (9), which is required for its nuclear transport (18). As shown here, in cells exposed to 17DMAG, there was a reduction in the radiation-induced interaction between ErbB1 and DNA-PKcs as well as in the radiation-induced activation of DNA-PKcs. These data thus suggest that the 17DMAG-mediated reduction in ErbB1 activity attenuates

the radiation-induced interaction between ErbB1 and DNA-PKcs leading to a reduction in DNA-PKcs activation after irradiation, consequently compromising the repair of DSBs.

Whereas 17DMAG was clearly shown to inhibit the repair of radiation-induced DSBs, a previous study using DU145 cells suggested that it also prevents the activation of the G₂- and S-phase checkpoints (4). The data presented here using MiaPaca cells confirm that 17DMAG exposure, and thus Hsp90 inhibition, abrogates the radiation-induced activation of the G₂- and S-phase checkpoints. Investigations into the mechanism responsible included an assessment of the potential contribution of ATM, which plays a critical role in the activation of the G₂- and S-phase checkpoints and is a critical regulator of cellular radiosensitivity (22). Although the levels of radiation-induced, phosphorylated ATM were reduced in cells exposed to 17DMAG as was the number of phospho-ATM-containing nuclear foci, no interaction between ATM and Hsp90 was detected, suggesting that this effect was mediated by other proteins or processes requiring Hsp90 activity. Recent studies have indicated that the MRN complex (MRE-11, RAD50, and NBS1) is not only a substrate for ATM, but also serves as a primary sensor of DSBs and participates in ATM activation (25, 36). More specifically, NBS1 has been implicated in mediating the recruitment of the MRN complex to the site of DNA damage and to be essential for the optimal activation of ATM (24, 25, 37–39). Indeed, the carboxyl terminus of NBS1 has been shown to directly interact with ATM, contributing to its activation

(26, 30, 39). In contrast to ATM, NBS1 does seem to be an Hsp90 client in MiaPaca cells; moreover, treatment with 17DMAG disrupted the interaction between NBS1 and ATM. At this point, it is not possible to discern whether NBS1 and/or the other MRN components are Hsp90 clients. Regardless, the critical interaction between NBS1 and ATM is disrupted by Hsp90 inhibition. Thus, these data suggest that the 17DMAG-mediated abrogation of the G₂- and S-phase cell cycle checkpoints is the result of a disruption of the NBS1/ATM interaction, which leads to a diminished activation of ATM after irradiation.

Most studies related to cancer treatment have focused on cytoplasmic Hsp90 and its role in the stabilization of various kinases. However, Hsp90 is also found in the nucleus, albeit at considerably smaller levels (40–42). The function of nuclear Hsp90 has not been clearly defined, although it has been shown to participate in the translocation into and retention of steroid receptors in the nucleus as well as the regulation of gene transcription (43–45). The data presented here indicates that nuclear Hsp90 is also bound to the MRN complex. It should be noted that because of the vastly greater levels of Hsp90 in the cytoplasm, similar experiments using whole cell extracts do not yield detectable interactions between these proteins (data not shown). Exposure of MiaPaca cells to 17DMAG compromised the ability of the MRN components to form nuclear foci and diminished the interaction of NBS1 and ATM. However, there was no detectable loss (degradation) of any of the MRN components nor did 17DMAG disrupt the complex. Ohji et al. (46) reported that raptor is an Hsp90 client, and although inhibition of Hsp90 does not result in raptor degradation, it does suppress raptor-mammalian target of rapamycin signaling. Similarly, the activity of the MRN complex may require a stabilizing interaction with Hsp90. Although the specific mechanism remains to be determined, these data suggest a role for Hsp90 in MRN function, at least in cells radiosensitized by 17DMAG.

Although the 17DMAG-mediated inhibition of Hsp90 attenuates both the repair of DSBs and the activation of cell cycle checkpoints, whether both effects are necessary and/or sufficient for 17DMAG-induced radiosensitization remains to be determined. The abro-

gation of cell cycle checkpoints alone leading to enhanced radiosensitivity has been questioned based on genetic experiments (47, 48) and the inconsistent radiosensitization induced by pharmacologic inhibitors of G₂ arrest (49, 50). In contrast, DSB repair is an established determinant of radiosensitivity; inhibition of critical components such as DNA-PK, genetically or pharmacologically, consistently induces radiosensitization (19). However, the effects of 17DMAG on DNA-PKs activation, was not a complete abrogation, merely a reduction in degree suggesting that Hsp90 (and thus ErbB1) is not an absolute requirement but acts to facilitate DNA-PK activity. Along these lines, we had previously shown that the reduction in ErbB1 activity alone using small interfering RNA did not affect MiaPaca cell radiosensitivity (9).

MRN, in addition to cell cycle checkpoints, via its relationship with ATM, has also been implicated in the repair of small fraction of DSBs detectable as γ H2AX foci remaining at 24 hours postirradiation (48). As for DNA-PKs, ATM activation in 17DMAG-treated MiaPaca cells was attenuated but not totally eliminated. Thus, in cell lines susceptible to 17DMAG-induced radiosensitization, Hsp90 seems to facilitate the optimal activation of DNA-PKs and MRN/ATM; the DSB repair defect may then reflect the combination of effects. Furthermore, the abrogation of the G₂- and S-phase checkpoints, which allow cells to enter mitosis and proceed with DNA synthesis, respectively, in the presence of genomic injury, may contribute to the full expression of the attenuated DNA repair process. Clearly, this is speculation and the specific contribution of each of these effects to 17DMAG-induced radiosensitization requires further investigation. Moreover, although the data presented here do indicate that Hsp90 can play a role in DSB repair and cell cycle checkpoint activation, the cellular circumstances that incorporate this chaperone into an optimal DNA damage response to radiation remains a critical question.

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