Effect of combined DNA repair inhibition and G_2 checkpoint inhibition on cell cycle progression after DNA damage

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Abstract

In response to DNA damage, cell survival can be enhanced by activation of DNA repair mechanisms and of checkpoints that delay cell cycle progression to allow more time for DNA repair. Inhibiting both responses with drugs might cause cancer cells to undergo cell division in the presence of lethal amounts of unrepaired DNA. However, we show that interfering with DNA repair via inhibition of DNA-dependent protein kinase (DNA-PK) reduces the ability of checkpoint inhibitors to abrogate G_2 arrest and their radiosensitizing activity. Cells exposed to the DNA-PK inhibitor AMA37, DNA-PK–deficient cells, and nonhomologous end joining–deficient cells all enter prolonged G_2 arrest after exposure to ionizing radiation doses as low as 2 Gy. The checkpoint kinase Chk2 becomes rapidly and transiently overactivated, whereas Chk1 shows sustained overactivation that parallels the prolonged accumulation of cells in G_2. Therefore, in irradiated cells, DNA repair inhibition elicits abnormally strong checkpoint signaling that causes essentially irreversible G_2 arrest and strongly reduces the ability of checkpoint kinase inhibitors to overcome G_2 arrest and radiosensitize cells. Variable levels of proteins controlling DNA repair have been documented in cancer cells. Therefore, these results have relevance to the development of DNA-PK inhibitors and G_2 checkpoint inhibitors as experimental therapeutic approaches to enhance the selective killing of tumor cells by radiotherapy or DNA-damaging chemotherapeutic agents. [Mol Cancer Ther 2006;5(4):885–92]

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Introduction

DNA damage can elicit a variety of cellular responses (1). Some, such as apoptosis, mitotic catastrophe, and premature senescence, remove damaged cells from proliferating populations. Others, such as DNA repair and cell cycle checkpoints, which delay cell cycle progression and allow more time for DNA repair, promote cell survival and subsequent proliferation. Considerable efforts are being invested into identifying chemicals that modulate these responses and investigating whether they have potential as cancer therapy agents. In particular, it has been proposed that drugs that inhibit DNA repair or cell cycle checkpoints might enhance the efficacy of current DNA-damaging cancer treatments, such as ionizing radiation and many chemotherapeutic drugs.

According to current models, DNA damage activates the phosphoinositide 3-kinase–like kinase family members DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM), and ataxia telangiectasia and rad3 related (ATR). Repair of double-strand breaks, the most lethal DNA lesions caused by ionizing radiation, is accomplished through two major pathways: nonhomologous end joining (NHEJ) and homologous recombination repair (2). NHEJ is a G_1-S-predominant process controlled by DNA-PK (a complex of the catalytic subunit DNA-PK_{CS}, Ku70/86), DNA Ligase IV, Artemis, and XRCC4 (3). Homologous recombination repair is a late S-G_2-predominant process that requires Rad52, Rad54, and various Rad51 paralogues (4, 5). ATM and ATR phosphorylate and activate the checkpoint kinases Chk1 and Chk2, which phosphorylate a variety of downstream targets resulting in cell cycle delay at the G_1 and G_2 phases of the cell cycle and slow down of S-phase traversal.

More than 50% of human cancers have a mutated form of p53, resulting in an inoperative G_1 checkpoint (6) but a functional, albeit weakened G_2 checkpoint. Some inhibitors of the G_2 checkpoint can selectively sensitize p53-defective cells to DNA-damaging agents, likely as a result of undergoing mitosis in the presence of damaged DNA (reviewed in ref. 7). DNA-PK–defective cells show increased sensitivity to ionizing radiation, and it was recently shown that DNA-PK inhibitors can radiosensitize tumors *in vitro* and *in vivo* (8, 9).

We reasoned that cells treated with ionizing radiation and a DNA-PK inhibitor would arrest in G_2 phase with more unrepaired DNA than cells exposed to irradiation alone. Exposure of these G_2-arrested cells to checkpoint inhibitors would then force them to divide in the presence of more heavily damaged DNA, which might translate into increased killing. However, in the absence of NHEJ repair, DNA damage caused a stronger activation of checkpoint kinases and a more pronounced and prolonged cell cycle arrest, which G_2 checkpoint inhibitors were less able to

overcome and increase cell killing. These observations are relevant to efforts to develop checkpoint inhibitors and DNA-PK inhibitors as tumor-selective radiosensitizers for cancer therapy.

Materials and Methods

Cell Lines, Irradiation, and Checkpoint Inhibitor Treatment

MO59K, MO59J, and MO59J/Fus1 cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD). MO59J/Fus1 cells were additionally supplemented with 250 μg/mL G418 (Invitrogen, San Diego, CA). All Chinese hamster ovary (CHO) cells were cultured in DMEMα with 10% fetal bovine serum. CHO K1 and XRCC4 cells were obtained from the Coriell Cell Repository (Camden, NJ). CHO V3 cells were transfected with either empty vector or hDNA-PK_{CS} cDNA (10) and were a gift from Susan Lees-Miller. CHO 51D1 and 51D1.3 cells were a gift from Larry H. Thompson. MCF-7 mp53 cells were cultured as previously described (11, 12). Cells were irradiated at a rate of 0.75 Gy/min using a ^{60}Co source (Gammacell 220, Atomic Energy Commission of Canada, Chalk River, Ontario, Canada). Caffeine (Sigma-Aldrich Co., St. Louis, MO), UCN-01 (National Cancer Institute), isogranulatimide (13), and debromohymenialdesine (14) were used to abrogate G_2 arrest, together with 300 ng/mL nocodazole (Sigma-Aldrich) to arrest cells in mitosis. AMA37 (Calbiochem, La Jolla, CA) was added to cells 30 minutes before treatment and refreshed every 8 hours.

Flow Cytometry and Checkpoint Inhibitor Assay

Cells were prepared for flow cytometry analysis exactly as described in (15). Checkpoint inhibition was assessed as described in ref. 15, except that CHO cells were irradiated with 6.5 Gy and incubated for 8 hours followed by checkpoint inhibitor plus nocodazole addition for an additional 4 hours.

Cell Clonogenicity Assays

To assess radiosensitization by checkpoint inhibitors and AMA37, MCF-7 cells were plated in serial dilutions into six-well plates. They were allowed to attach for 24 hours and then irradiated in the presence or absence of drug. After 24 hours, drugs were washed off, and cells were grown in fresh medium for 10 days. Colonies were stained with Malachite Green and counted. Clonogenicity was expressed as % colonies relative to untreated cells.

Kinase Assays

Following mock irradiation or exposure to 6.5 Gy, cells were further cultured as indicated. Cells were then harvested via trypsinization, washed twice in PBS, assayed for CDK1 (16), Chk1/2 (17), or ATM/ATR (18) as described with the following modifications. Protein concentration was determined using the Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL), with 125, 250, or 500 μg total protein lysate used for immunoprecipitation of CDK1, Chk1/2, or ATM/ATR, respectively. Antibodies used were 2 μg anti-ATR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1 μg anti-CDK1 antibody (KAP-CC001E, Stressgen), 1 μg anti-Chk2 antibody (Santa Cruz Biotechnology), 1 μg anti-Chk1 antibody (Santa Cruz Biotechnology), or 2 μg anti-ATM antibody (Ab-3 PC116, Oncogene, Uniondale, NY). Substrates used were 1 μg histone H1 (Sigma, St. Louis, MO), 1 μg GST-$CDC25C_{200-256}$ (19), or 2 μg PHAS-1 (AG Scientific, San Diego, CA) for CDK1, Chk1/2, or ATM/ATR assays, respectively. Samples were separated via SDS-PAGE, exposed to Kodak X-OMAT AR film, and developed according to the manufacturer's instructions.

Results and Discussion

DNA-PK Inhibition and NHEJ Deficiency Decrease the Ability of Cancer Cells to Respond to G_2 Checkpoint Inhibitors

Caffeine, an inhibitor of ATM and ATR (20, 21), and UCN-01, isogranulatimide, and debromohymenialdesine, inhibitors of Chk1 or Chk1/Chk2, can abrogate the G_2 checkpoint and potentiate the lethality of ionizing radiation (13, 14, 21–23). DNA-PK inhibitors can radiosensitize tumor cells by impairing DNA repair via the NHEJ system (8, 24). The arylmorpholine AMA37 is a potent *in vitro* inhibitor of DNA-PK (IC_{50} = 0.27 μmol/L) that does not inhibit ATM or ATR and inhibits phosphoinositide 3-kinases poorly (25). We wished to determine whether checkpoint inhibitors are able to overcome G_2 arrest in the presence of a DNA-PK inhibitor.

The interplay between DNA-PK inhibition and G_2 checkpoint inhibition was first examined in human breast carcinoma MCF-7 mp53 cells. These cells are NHEJ-proficient, have an inactive G_1 checkpoint (11, 26), and have been used extensively to study the G_2 checkpoint (11, 13–15). To measure G_2 checkpoint inhibition, MCF-7 mp53 cells were exposed to AMA37 or the drug carrier DMSO for 30 minutes and then irradiated with 6.5 Gy (Fig. 1A). At 16 hours, when G_2 arrest was maximal, cells were treated for 8 hours with 2 mmol/L caffeine, 100 nmol/L UCN-01, 100 μmol/L isogranulatimide, or 40 μmol/L debromohymenialdesine, together with nocodazole to trap cells that escaped G_2 arrest in mitosis. The cells were then analyzed by two-dimensional flow cytometry using the mitosis-specific antibody GF-7 to distinguish M cells from G_2 cells (ref. 15; see Supplementary Fig. S1[3] for examples). Quantitation of G_2 checkpoint inhibition after various treatments is presented in Fig. 1A. Caffeine, UCN-01, isogranulatimide, and debromohymenialdesine caused clear G_2 checkpoint inhibition. AMA37 reduced the ability of UCN-01, isogranulatimide, and debromohymenialdesine, but not caffeine, to overcome G_2 arrest ($\rho < 0.05$). By contrast, 200 nmol/L wortmannin, which inhibits phosphoinositide 3-kinases but not ATM or DNA-PK (27), did not reduce the activity of the checkpoint inhibitors (Fig. 1A). The effect of AMA37 on γ-H2AX phosphorylation was consistent with selective inhibition of DNA-PK *in vivo* (see Supplementary Fig. S2)[3],

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

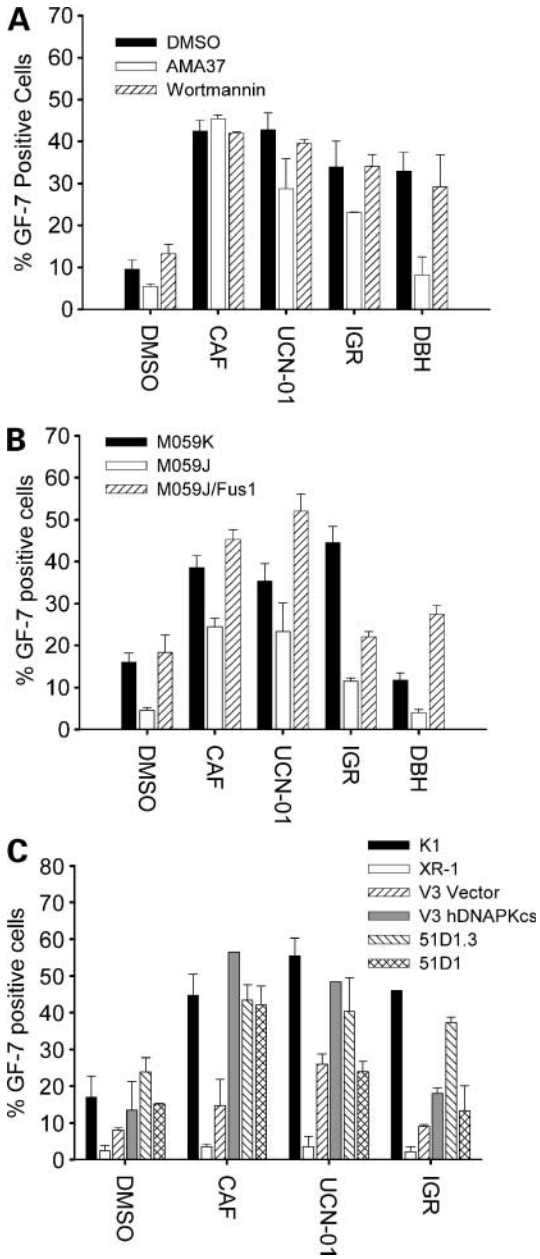

Figure 1. Effect of G_2 checkpoint inhibitors on cells lacking DNA-PK or NHEJ activity. **A,** MCF-7 mp53 cells were exposed to DMSO, 20 μmol/L AMA37, or 200 nmol/L wortmannin 30 min before irradiation with 6.5 Gy. Sixteen hours later, when arrested in G_2, cells were exposed to checkpoint inhibitors plus nocodazole for 8 h and harvested as described in Materials and Methods. GF-7-positive cells represent those trapped in mitosis. **B,** MO59 cells were irradiated and treated as in **A**. **C,** CHO cells were irradiated with 6.5 Gy. Eight hours after ionizing radiation, checkpoint inhibitors and nocodazole were added for 4 h. *Columns,* average between different experiments ($n \geq 2$); *bars,* SD.

indicating that the effects of AMA37 were likely due to DNA-PK inhibition and not to modulation of other phosphoinositide 3-kinase–like kinase involved in the checkpoint response.

This experiment was prompted by the premise that G_2 checkpoint inhibitors can induce cells to enter mitosis in the presence of DNA damage and that cotreatment with a DNA-PK inhibitor to reduce DNA repair would cause cells to enter mitosis with even more damaged DNA. It was surprising that cells treated with AMA37 showed a reduced response to G_2 checkpoint inhibitors. To further investigate whether the effect was due to reduced DNA-PK activity, we examined the response of human cells lacking the DNA-PK_{CS} to checkpoint inhibitors. Three related cell lines obtained from a human malignant glioma were used (28, 29). DNA-PK-deficient MO59J cells fail to express the DNA-PK_{CS}, whereas DNA-PK-proficient MO59K cells do express it (28, 30). However, MO59J cells express lower levels of ATM than MO59K (31–33). MO59J/Fus1 cells, rescued in their expression of DNA-PK_{CS} (29), have slightly higher ATM expression levels than MO59J and are the closest available genetic match to MO59J (29). The three cell lines are p53 deficient (26, 34, 35). As shown in Fig. 1B, caffeine, UCN-01, and isogranulatimide caused clear G_2 checkpoint inhibition in MO59K cells. However, MO59J cells were less responsive to the checkpoint inhibitors. Furthermore, MO59J/Fus1 cells were more responsive to all inhibitors than MO59J cells (Fig. 1B). Interestingly, at a higher dose of 10 Gy, MO59K cells responded as in Fig. 1B, whereas both MO59J and MO59J/Fus1 failed to respond to checkpoint inhibitors (data not shown). No cell death was observed for any of the cell lines or treatments during the course of these experiments.

These data indicated that DNA-PK activity is required for cells to resume cell cycle progression after G_2 arrest, or that the reduced activity of checkpoint inhibitors is a consequence of reduced DNA repair via NHEJ in cells lacking DNA-PK activity. To address this issue, we monitored checkpoint inhibition in an additional six related CHO cell lines. CHO K1 cells are considered wild type. CHO XR-1 cells do not express XRCC4, making them defective for NHEJ repair (36). CHO V3 cells are deficient in DNA-PK_{CS} (37). CHO V3 cells stably transfected with either empty vector (CHO V3) or human DNA-PK_{CS} cDNA (CHO V3 hDNA-PK_{CS}) differ by the presence or absence of DNA-PK_{CS} (10). CHO 51D1 lack both alleles expressing Rad51D, whereas 51D1.3 are a Rad51D-reconstituted isogenic match. DNA-PK_{CS}-deficient V3 cells were less responsive to checkpoint inhibitors than their DNA-PK-proficient counterparts (Fig. 1C). Interestingly, XRCC4-deficient XR-1 cells were not responsive at all to checkpoint inhibitors, whereas K1 cells with functional NHEJ were. CHO 51D1 cells, deficient in Rad51D, were less responsive to UCN-01 and isogranulatimide, but not caffeine, than their Rad51D-expressing counterparts, 51D1.3. These data provide strong evidence that it is a lack of DNA repair that reduces the capacity of checkpoint inhibitors to abrogate G_2 arrest.

DNA-PK Inhibition Leads to Reduced Radiosensitization by Checkpoint Inhibitors

To determine whether the decreased ability of checkpoint inhibitors to abrogate G_2 arrest translates into

Table 1. Effect of AMA37 and checkpoint inhibitors on the clonogenicity of irradiated and unirradiated MCF-7 cells

Treatment	DMSO	Caffeine	UCN-01	Isogranulatimide
DMSO				
Mock	100	107	95.3	94.4
6.5 Gy	15.4 ± 2.0%	4.2 ± 0.7%	4.5 ± 1.1%	2.9 ± 1.2%
AMA37				
Mock	100	112.1	77.6	84.1
6.5 Gy	4.4 ± 1.6 %	1.0 ± 1.3 %	5.5 ± 0.7 %	3.6 ± 0.2 %

NOTE: Cells were treated with drug combinations at the time of irradiation and the % colonies present 10 days after ionizing radiation was determined relative to untreated controls.

reduced radiosensitization, we assessed the clonogenicity of MCF-7 mp53 cells following combined treatment with AMA37 and checkpoint inhibitors. Table 1 lists the effect of different drug combinations on clonogenicity. Exposure of cells to 6.5 Gy ionizing radiation alone lead to a decrease in survival to 15.4 ± 2.0% of unirradiated controls. Irradiation in the presence of caffeine, UCN-01, or isogranulatimide caused a further decrease in clonogenicity, indicating radiosensitization. Inhibition of DNA-PK with AMA37 also lead to radiosensitization. The checkpoint kinase inhibitors UCN-01 and isogranulatimide were unable to radiosensitize cells in the presence of AMA37, consistent with their inability to overcome G_2 arrest. Caffeine, the only compound that retained checkpoint inhibitory activity in the presence of AMA37 (Fig. 1A), was also able to radiosensitize in the presence of AMA37. Caffeine inhibits ATM and ATR and acts upstream of the Chk kinase inhibitors UCN-01 and isogranulatimide. Caffeine is also a less selective checkpoint inhibitor than UCN-01 and isogranulatimide. It inhibits several other cellular activities, including DNA repair itself (reviewed in ref. 7). We speculate that caffeine's radiosensitizing effect in the presence of AMA37 may due to its inhibition of DNA repair and/or its ability to down-regulate more branches of the checkpoint response than UCN-01 and isogranulatimide.

DNA-PK- and NHEJ-Impaired Cells Enter Prolonged G_2 Arrest following DNA Damage

G_2 checkpoint inhibitors can force entry into mitosis in the presence of DNA damage (13, 14, 21, 22). To investigate why cells with reduced DNA repair were less responsive to checkpoint inhibitors, we used flow cytometry to monitor G_2 arrest over time in the cell lines examined above. As expected, MCF-7 mp53 cells exposed to ionizing radiation underwent transient G_2 arrest (Fig. 2A; see Supplementary Fig. S3 for example flow profiles).[3] However, treatment with AMA37 and ionizing radiation resulted in a higher proportion of cells arresting in G_2 and their failure to exit spontaneously from G_2 arrest (Fig. 2A). Cells treated with 200 nmol/L wortmannin and ionizing radiation underwent G_2 arrest and subsequently exited G_2 arrest similarly to untreated cells (Fig. 2A), indicating that AMA37 does not affect the G_2 checkpoint via phosphoinositide 3-kinase inhibition. LY294002, which inhibits phosphoinositide 3-kinases and DNA-PK but not ATM or ATR *in vivo* (38), also leads to prolonged G_2 arrest after ionizing radiation. These results indicated that inhibition of DNA-PK causes a prolonged G_2 arrest. Consistent with NHEJ's role in DNA repair in G_1-S, adding AMA37 to G_2-arrested cells (16 hours after ionizing radiation) did not induce prolonged G_2 arrest (Fig. 2A), indicating that AMA37 must exert its effects before G_2 arrest becomes established, and that DNA-PK activity is not required for cells to resume cell cycle progression after G_2 arrest.

To ensure that the arrest was in G_2 and not late S phase, we used flow cytometry to monitor the presence of the G_2 marker cyclin B1. Cyclin B1 accumulated in cells with 4*n* DNA content, and its levels remained elevated in AMA37-treated MCF-7 cells during prolonged arrest (see example profiles in Supplementary Fig. S3C).[3] Similar results were obtained with Western blotting (data not shown). This result indicates that the cells were indeed arrested in G_2 phase and not in late S-phase or failed cytokinesis. The observation that cyclin B1 levels are stable during prolonged G_2 arrest also indicates that the checkpoint inhibitors did not fail to overcome G_2 arrest because of down-regulation of cyclin B1.

Quantitation of the G_2-M peaks as a function of time after irradiation of MO59 cells is shown in Fig. 2B. Exposure to 6.5 Gy caused a large increase in the G_2-M peak that was maximal at 16 hours for all three cell lines. Most of the MO59K cells escaped G_2 arrest between 16 and 24 hours and cycled thereafter as a loosely synchronous population. Similar to AMA37 treatment, MO59J cells remained arrested in G_2 for at least 48 hours after ionizing radiation. Cell populations with sub-G_1 DNA content became increasingly evident at time points longer than 48 hours, indicating the onset of cell death (data not shown). Cyclin B1 also accumulated in G_2-arrested MO59J cells (Supplementary Fig. S3C).[3] MO59J/Fus1 cells began to exit G_2 arrest 16 hours after exposure 6.5 Gy, much like MO59K cells (Fig. 2B). Additional ionizing radiation doses were tested in these cell lines: a lower dose of 2 Gy caused a significant increase in the G_2-M peak only in MO59J cells and remained for upwards of 48 hours. A higher dose of 10 Gy caused a large increase in G_2-M that peaked at 16 to 24 hours in all cell lines, with MO59K and MO59J/Fus1 cells recovering from this arrest. However, MO59J/Fus1

cells remained arrested longer and fewer cells finally exited G_2 arrest at this dose ($P < 0.0001$; see Supplementary Fig. S3A).[3] Interestingly, MO59J/Fus1 cells have about 60% of the DNA-PK activity of MO59K (29), and they exited G_2 arrest significantly later and to a lesser extent than M059K after exposure to 10 Gy but not lower doses, suggesting a relationship between the level of cellular DNA-PK activity and the ability of cells to escape G_2 arrest. At 10 Gy, MO59J/Fus1 cells were equally unresponsive to checkpoint inhibitors as MO59J (data not shown), whereas MO59K cells remained equally responsive.

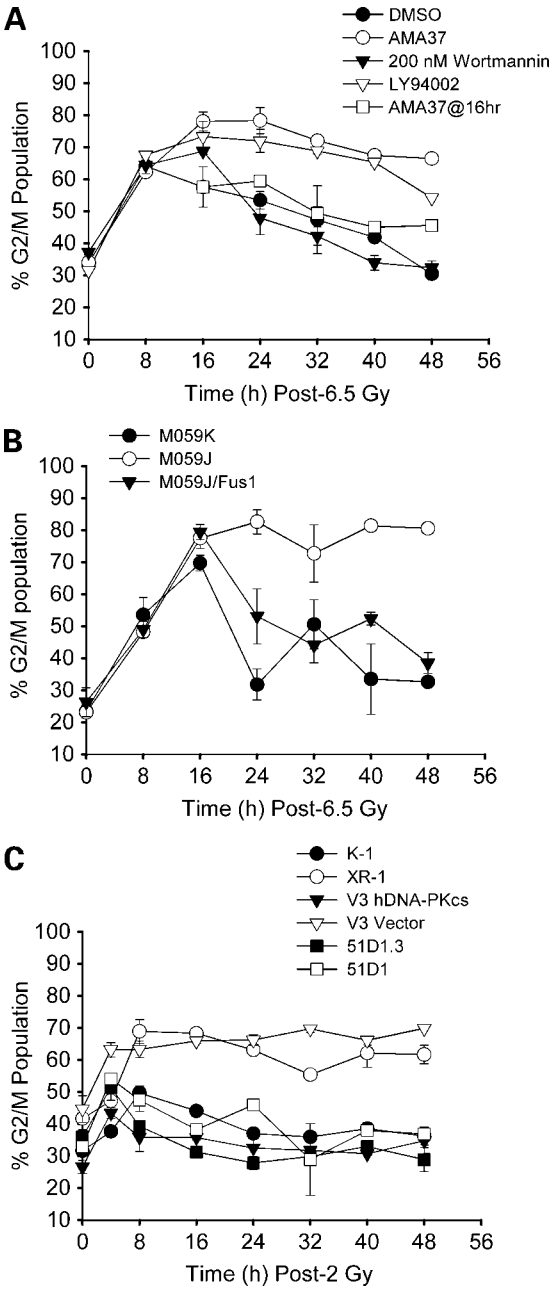

Figure 2. Effect of ionizing radiation on G_2 arrest in cells lacking DNA-PK or NHEJ activity. Cells were irradiated at time 0 and sampled at the indicated times, and the G_2-M populations were quantitated using flow cytometry. **A,** MCF-7 mp53 cells were exposed to DMSO, 20 μmol/L AMA37, 200 nmol/L wortmannin, or 50 μmol/L LY294002 30 min before irradiation with 6.5 Gy. Another cell population was irradiated with 6.5 Gy, and AMA37 was added 16 h later. Cells were harvested at the indicated times and analyzed by flow cytometry. **B,** MO59K, MO59J, or MO59J/Fus1 cells were exposed to 6.5 Gy. **C,** CHO cells were exposed to 2 Gy. *Points,* average between different experiments ($n \geq 3$); *bars,* SD.

The different CHO cell lines were subjected to irradiation with 2 Gy, and G_2 arrest was monitored over time. Ionizing radiation elicited a transient G_2 arrest in K1 cells and V3 hDNA-PK_{CS} cells, both of which have DNA-PK_{CS} and NHEJ activity (Fig. 2C). However, DNAPK_{CS}-defective V3 cells and NHEJ-defective XR-1 cells showed strong sustained G_2 arrest (Fig. 2C). Exit from G_2 arrest started after 8 hours in K1 and V3 hDNA-PK_{CS} cells, whereas V3 and XR-1 cells remained arrested in G_2 for at least 48 hours. For unclear reasons, G_2 arrest was also accompanied by an increase in $\geq 8n$ population in XR-1 and V3 cells, and a large increase in apoptosis was observed at the higher dose of 6.5 Gy (see Supplementary Fig. S3D).[3] The G_2 arrest profile of 51D1 and 51D1.3 cells irradiated with 2 Gy was similar to that of wild-type K1 cells (Fig. 2C), but 6.5 Gy caused a slightly prolonged G_2 arrest in 51D1 cells (see Supplementary Fig. S3D).[3] Together, these results show that lack of repair at the time of DNA damage causes prolonged G_2 arrest that cannot be abrogated by checkpoint inhibitors, likely caused by the persistence of DNA lesions.

Ionizing Radiation Causes Overactivation of Chk1 and Chk2 in Cells Lacking DNA-PK Activity

We next wished to examine the mechanism underlying prolonged G_2 arrest in cells lacking DNA repair. The G_2 checkpoint is generally believed to comprise two convergent pathways: ATM activation followed by Chk2 activation and ATR activation followed by Chk1 activation, both leading to CDC25 and CDK1 inhibition (39, 40). We therefore monitored the activation of the canonical checkpoint pathways using *in vitro* kinase assays.

We first measured the activity of the kinases ATR, Chk1, and CDK1 in M059 cells. Cells were exposed or not to 6.5 Gy and incubated for 16 hours to achieve maximal G_2 arrest, and the activity of the immunoprecipitated kinases was measured *in vitro* using substrate proteins or peptides (see Materials and Methods). The protein levels of ATR, Chk1, and CDK1 ($p34^{cdc2}$) were similar in the three cell lines and did not change after irradiation (Fig. 3A). Ionizing radiation caused an increase in ATR activity in MO59K and MO59J/Fus1 cells but not in MO59J (Fig. 3A). No increase in Chk1 kinase activity was observed in MO59K or MO59J/Fus1 cells following ionizing radiation. However, a clear increase in Chk1 kinase activity was observed in irradiated MO59J cells (Fig. 3A), which required several hours after ionizing radiation to become apparent. This result was unexpected because Chk1 is typically activated poorly by ionizing radiation (40), and the upstream activator of Chk1 is ATR (41). As expected, CDK1 kinase activity decreased following DNA damage in all cell lines, but the decrease was slightly stronger in MO59J cells (Fig. 3A), likely due to increased

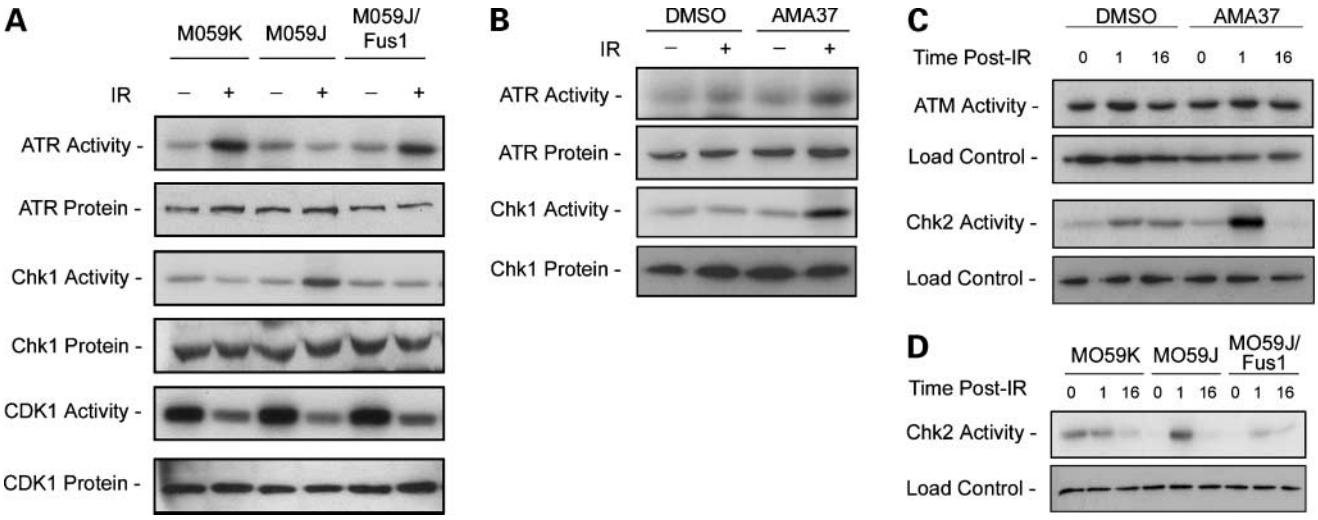

Figure 3. Overactivation of Chk1 and Chk2 in DNA-PK-deficient cells. **A,** ATR, Chk1, and CDK1 activity in irradiated cells. Cells were harvested 16 h after mock irradiation (−) or exposure to 6.5 Gy (+). Cellular levels of ATR, Chk1, and CDK1 protein were measured by Western blotting, and their kinase activity was measured in immunoprecipitates as described in Materials and Methods. **B,** MCF-7 mp53 cells were treated without or with AMA37 30 min before irradiation, and cellular levels and kinase activity of ATR and Chk1 were measured as above 16 h after irradiation. **C,** ATM and Chk2 activity in irradiated MCF-7 mp53 cells harvested 1 or 16 h after ionizing radiation (*IR*). **D,** Chk2 kinase activity of MO59 cells measured at 1 or 16 h after ionizing radiation.

Chk1 activity. Interestingly, an ionizing radiation dose of 50 Gy was required to elicit a similarly high level of Chk1 activation in DNA-PK-complemented MO59J/Fus1 cells (data not shown).

We also tested the effect of AMA37 on Chk1 activity in DNA-PK-proficient MCF-7 mp53 cells. The cells were preincubated with AMA37 for 30 minutes, irradiated or not, and the protein levels and kinase activity of ATR and Chk1 were measured after 16 hours, when G_2 arrest was maximal. Following ionizing radiation, Chk1 activity increased strongly in cells treated with AMA37 but not in cells treated with DMSO (Fig. 3B). ATR activity also increased but to a lesser extent (Fig. 3B).

We next examined the activity of the kinases ATM and Chk2. MCF-7 mp53 cells were preincubated with AMA37 and irradiated. One hour after exposure to 6.5 Gy, ATM activity in both DMSO- and AMA37-treated cells increased slightly and then decreased by 16 hours (Fig. 3C). In the absence of AMA37, Chk2 activity was increased at both 1 and 16 hours after irradiation. However, very strong Chk2 activity was observed in AMA37-treated cells 1 hour after ionizing radiation (Fig. 3C). To confirm that this strong transient increase was indeed due to lack of DNA-PK activity, M059K, M059J, and M059J/Fus1cells were irradiated, and Chk2 activity was measured 1 and 16 hours later. Chk2 was clearly overactivated in DNA-PK-deficient M059J cells compared with DNA-PK-complemented MO59J/Fus1 cells and M059K cells (Fig. 3D). We were unable to examine checkpoint kinase activity in CHO cells because available antibodies showed insufficient selectivity towards rodent proteins.

Overall, the results show that irradiation of cells lacking DNA-PK activity or cells exposed to the DNA-PK inhibitor AMA37 causes overactivation of Chk1 and Chk2. This abnormally high checkpoint signaling is likely the mechanism underlying the prolonged G_2 arrest. Prolonged G_2 arrest dependent on Chk1 overactivation has also been observed in human AT cells after DNA damage, and this requires S-phase traversal (42), as well as in mouse Ku86-deficient cells (19). The replication of damaged DNA in S phase could generate secondary lesions that overactivate the checkpoint in G_2 (42). This explanation likely also applies to cells lacking DNA-PK activity. In addition, we observed that cells lacking DNA-PK activity showed abnormally high Chk2 activity within 1 hour of irradiation. In normal cells, Chk2 activation is probably limited because rapid DNA repair restricts the magnitude of the damage signal.

The overactivation of Chk1 and Chk2 in cells lacking DNA-PK activity also adequately explains why cells show a reduced response to G_2 checkpoint inhibitors that target Chk1 and/or Chk2. At concentrations that can be used on cells, these inhibitors may not counteract the overactivated pathway sufficiently to override G_2 arrest. There may also be additional differences between cells undergoing transient G_2 arrest and cells undergoing prolonged G_2 arrest that render checkpoint inhibitors less effective. At time points longer than 48 hours, most G_2-arrested MO59J cells showed sub-G_1 DNA peaks, indicating apoptosis. However, many surviving cells were observed, and they showed signs of senescence, including a large size, flattened shape, and increased acidic β-galactosidase activity (data not shown). Recently, it has been shown that senescent human fibroblasts exhibit similar markers to DNA damage checkpoint-arrested cells, such as colocalization of phosphorylated H_2AX with 53BP1, MDC1, and NBS1, along with overactivation of the

checkpoint kinases Chk1/Chk2 (43). Below a threshold level of G_2 checkpoint signaling, cells may undergo transient G_2 arrest to increase time for DNA repair and then resume cell cycle progression, whereas above this threshold, cells may arrest permanently and enter a senescent state that is unresponsive to G_2 checkpoint inhibitors. Drugs targeting DNA-PK might be useful to drive cancer cells into a senescent state.

Variable levels of DNA-PK activity and DNA-PK_{CS}, Ku70, and Ku86 protein levels have been documented in a variety of cancers (44–48). The observation that the level of DNA-PK activity influences the ability of cells to resume cell cycle progression after exposure to a clinical dose of 2 Gy may, therefore, have relevance to the fate of tumor cells after radiotherapy or treatment with DNA-damaging chemotherapeutic agents, and ultimately to the clinical outcome of these therapeutic approaches. In addition, combination therapy with DNA-damaging agents and G_2 checkpoint inhibitors or DNA-PK inhibitors has been proposed as a way to increase the selective killing of tumor cells (7–9, 24). The observation that DNA repair inhibition strongly affects the response of cells to checkpoint inhibitors should be taken into consideration when further developing these experimental therapeutic approaches.

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