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Inhibition of ATM and ATR Kinase Activities by the Radiosensitizing Agent, Caffeine[1]

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ABSTRACT

Caffeine exposure sensitizes tumor cells to ionizing radiation and other genotoxic agents. The radiosensitizing effects of caffeine are associated with the disruption of multiple DNA damage-responsive cell cycle checkpoints. The similarity of these checkpoint defects to those seen in ataxia-telangiectasia (A-T) suggested that caffeine might inhibit one or more components in an A-T mutated (ATM)-dependent checkpoint pathway in DNA-damaged cells. We now show that caffeine inhibits the catalytic activity of both ATM and the related kinase, ATM and Rad3-related (ATR), at drug concentrations similar to those that induce radiosensitization. Moreover, like ATM-deficient cells, caffeine-treated A549 lung carcinoma cells irradiated in G_2 fail to arrest progression into mitosis, and S-phase-irradiated cells exhibit radioresistant DNA synthesis. Similar concentrations of caffeine also inhibit γ- and UV radiation-induced phosphorylation of p53 on Ser^{15}, a modification that may be directly mediated by the ATM and ATR kinases. DNA-dependent protein kinase, another ATM-related protein involved in DNA damage repair, was resistant to the inhibitory effects of caffeine. Likewise, the catalytic activity of the G_2 checkpoint kinase, hChk1, was only marginally suppressed by caffeine but was inhibited potently by the structurally distinct radiosensitizer, UCN-01. These data suggest that the radiosensitizing effects of caffeine are related to inhibition of the protein kinase activities of ATM and ATR and that both proteins are relevant targets for the development of novel anticancer agents.

INTRODUCTION

Cell cycle checkpoints are signal transduction pathways that insure the timing, sequence, and fidelity of critical cell cycle events and orchestrate cellular responses to environmentally induced genotoxic stress. DNA damage induced by ionizing radiation or other insults triggers checkpoint activation and consequent cell cycle arrest, followed by DNA repair or apoptosis. Disruption of cell cycle checkpoints leads to increased genomic instability, which predisposes an organism to the development of cancer. The tumor suppressor protein, p53, plays a central role in the function of the DNA damage-induced G_1-phase checkpoint (1, 2). Cellular exposure to a variety of genotoxic stresses induces the rapid phosphorylation and acetylation of p53 (3, 4). These modifications result in the accumulation and functional activation of p53. The high frequency of p53 mutations in human tumors highlights the importance of p53-dependent checkpoint functions in suppressing cancer development and/or progression.

Accumulating data suggest that several members of the PIKK[4] family play pivotal roles in cell cycle checkpoint functions in eukaryotic cells (5, 6). The PIKK family members share a COOH-terminal kinase domain bearing significant sequence homology to the catalytic domains of mammalian and yeast phosphoinositide 3-kinases. Genetic and biochemical evidence indicates that several of the PIKK family members, including yeast Rad3p and Mec1p, and the mammalian proteins, ATM and ATR, are proximal components of DNA damage-induced, cell cycle checkpoint pathways. The importance of ATM in the regulation of cell viability and genomic stability in humans is dramatically highlighted by the severe pathologies displayed by patients with the heritable disorder, A-T, which results from mutational inactivation of both *ATM* alleles. The A-T syndrome is characterized by progressive cerebellar ataxia and neurodegeneration, immunodeficiency, heightened cancer susceptibility, and a marked increase in cellular sensitivity to ionizing radiation (7, 8). Cells derived from A-T patients are defective in the activation of G_1, S, and G_2 cell cycle checkpoints after exposure to ionizing radiation. These pleiotropic checkpoint abnormalities presumably underlie the radiation hypersensitivity and chromosomal instability displayed by A-T cells (9, 10).

A second mammalian PIKK, ATR, carries out checkpoint-related functions that partially overlap with those performed by ATM. Both ATM and ATR contribute to the phosphorylation and accumulation of p53 after cellular exposure to γ- or UV-radiation. These DNA-damaging agents induce the phosphorylation of p53 on Ser^{15}, and genetic manipulations that reduce the activity of either ATM or ATR decrease Ser^{15} phosphorylation in irradiated cells (11, 12). Furthermore, both ATM and ATR phosphorylate p53 at Ser^{15} in immune complex kinase assays (11, 13, 14). Recent findings suggest that ATM may be the major Ser^{15} kinase in γ-irradiated cells, whereas ATR plays a more prominent role in the phosphorylation of p53 at Ser^{15} in response to UV light exposure (3, 11).

Based in large part on studies of the homologous proteins in yeast, it is predicted that ATM and ATR function as proximal signal transducers in G_1, S, and G_2 checkpoint pathways. With the exception of p53, the downstream components of these pathways remain largely undefined. Studies in yeast and vertebrate cells have outlined a pathway whereby ATM and/or ATR might inhibit the passage of DNA-damaged cells from G_2 into M phase. DNA damage leads to the activation of two related protein kinases, Chk1 and Chk2, which then phosphorylate the Cdc25C phosphatase on Ser-216. This modification creates a binding site for 14-3-3 proteins (15–18). The 14-3-3-bound phosphatase is then sequestered outside of the nucleus and is prohibited from dephosphorylating and activating the mitosis-promoting cyclin B-cdc2 complex. In *Schizosaccharomyces pombe*, the ATM/ATR homologue, Rad3p, is required for the activation of spChk1 in

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[4] The abbreviations used are: PIKK, phosphatidylinositol 3-kinase related kinase; ATM, ataxia-telangiectasia mutated; ATR, ATM- and Rad3-related; A-T, ataxia-telangiectasia; RDS, radioresistant DNA synthesis; PLC, phospholipase C; HA, hemagglutinin; BrdUrd, bromodeoxyuridine; DNA-PK, DNA-dependent protein kinase; LLnL, *N*-acetyl-Leu-Leu-Norleucinal; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PMA, phorbol myristate acetate.

response to DNA damage, suggesting that ATM and/or ATR might carry out similar functions in mammalian cells (19).

A normal function of cell cycle checkpoints is to protect cells and the host organism from the deleterious consequences of replicating or segregating damaged chromosomes. Therefore, it seems paradoxical that p53-deficient, and hence G_1 checkpoint-compromised tumor cells, frequently show an elevated resistance to DNA-damaging therapeutic agents (20). These cells retain a functional G_2 checkpoint, and accumulating evidence suggests that this checkpoint, which is largely p53 independent, is critical for cellular recovery from DNA damage. Abrogation of the G_2 checkpoint often leads to a marked increase in the sensitivity of cells to ionizing radiation and certain chemotherapeutic agents (21, 22). These observations have provoked speculation that inhibitors of the G_2 checkpoint signaling pathway might effectively sensitize cancer cells to the lethal effects of ionizing radiation or DNA-damaging drugs. Indeed, two known inhibitors of cell cycle arrest at the G_2 checkpoint, caffeine and UCN-01 (7-hydroxystaurosporine), preferentially radiosensitize cells that lack functional p53 (23–26). The molecular targets for both drugs remain unclear. Nonetheless, the sensitizing actions of caffeine and UCN-01 have raised hopes that adjunctive therapy with G_2 checkpoint inhibitors will increase the therapeutic efficacies of radiation and other genotoxic therapies in the large population of cancer patients whose tumor cells lack functional p53.

The checkpoint defects induced by caffeine and other methylxanthines are reminiscent of the checkpoint defects seen in A-T cells. Exposure of cultured cells to caffeine results in a delayed and attenuated accumulation of wild-type p53 and abrogation of the G_1 checkpoint in response to ionizing radiation (27). Caffeine treatment also results in RDS, which represents a canonical radiation-response defect of A-T cells (28–30). On the basis of these similarities, we hypothesized that inhibition of PIKK-dependent signaling pathways could be responsible for the checkpoint defects observed in caffeine-treated cells. In this study, we show that caffeine inhibits ATM and ATR kinase activities at drug concentrations similar to those shown previously to sensitize cells to killing by ionizing radiation. Furthermore, caffeine-treated cells display several of the phenotypic abnormalities reported previously in cells deficient in ATM or ATR function. These data identify ATM and ATR as relevant molecular targets for caffeine and suggest that more potent and specific inhibitors of these PIKK family members might be clinically useful radio- and chemosensitizing agents.

MATERIALS AND METHODS

Cell Culture, Antibodies, and Plasmid Constructs. The A549 lung adenocarcinoma cell line and K562 erythroblastoid leukemia cell line were maintained in RPMI 1640 (Life Technologies, Inc.) medium containing 5% fetal bovine serum and 5% newborn bovine serum. Wortmannin (Sigma), staurosporine (Sigma) and UCN-01 (generously provided by Dr. Edward Sausville, National Cancer Institute) were dissolved in DMSO and stored at −80°C. Caffeine (Sigma) and pentoxifylline (Sigma) were dissolved in kinase buffer (see below) and stored at 4°C. Rabbit polyclonal antisera specific for mTOR, ATR, PLC-γ1, and phospho-Ser^{15}-p53 were generated as described (11, 31–33). Antibodies for ATM (Ab-3), DNA-PK (Ab-1), and p53 (Ab-6) were obtained from Oncogene Science-Calbiochem; phospho-specific mitogen-activated protein kinase (Erk-1 and Erk2) antibodies were obtained from New England Biolabs. The viral HA-specific mouse monoclonal antibody, HA.11, was purchased from Babco. For flow cytometry, a mouse anti-BrdUrd monoclonal antibody was obtained from Becton Dickinson, and a goat-anti-mouse antibody conjugated with FITC was obtained from Sigma. hChk1 was amplified by PCR from a human testes cDNA library (Clontech), and the full-length cDNA was cloned into the pEF-BOS plasmid with a COOH-terminal HA_2 epitope tag. To generate a GST fusion protein containing amino acids 200–256 of Cdc25C (GST-$Cdc25C^{200-256}$), the corresponding cDNA fragment was amplified by PCR from a human testes cDNA library and cloned into the pGEX-KG vector.

Immune Complex Kinase Assays. The ATM and DNA-PK proteins were immunoprecipitated from 0.2% Tween 20 extracts prepared from A549 cells (34). The modifications to the procedure reported previously were that the lysis buffer was modified by addition of 10 mM sodium fluoride and 20 mM β-glycerophosphate, and that, prior to immunoprecipitation of ATM, the cells were subjected to two freeze-thaw cycles in a dry ice-ethanol bath. In the indicated experiments, immunoprecipitated proteins were incubated for 15 min on ice with 2× final concentrations of caffeine or other checkpoint inhibitors diluted in kinase base buffer (10 mM HEPES, 50 mM NaCl, and 10 mM $MgCl_2$, pH 7.4). The kinase reactions were then performed as detailed previously (34). Kinase reactions were terminated by the addition of 4× SDS sample buffer, and reaction products were resolved by SDS-PAGE. Incorporation of $^{32}P_i$ into the PHAS-I substrate was quantitated by phosphorimaging.

ATR and mTOR immunoprecipitations were performed as described (11, 32). ATR was immunoprecipitated from bull testis extract, and mTOR was immunoprecipitated from rat brain extract. The immune complexes were incubated with graded concentrations of caffeine as described above. The kinase reactions were then performed using kinase reaction components and conditions that were identical to those used in the ATM kinase reactions. All kinase reactions were performed under linear reaction conditions.

The hChk1 kinase assays were performed with recombinant epitope-tagged hChk1. K562 cells were transfected with 5 μg of pEF-BOS-hChk1-HA_2 by electroporation using conditions described previously (11). After 18–20 h, the transfected cells were lysed in buffer containing 50 mM HEPES (pH 7.6), 150 mM sodium chloride, 10 mM sodium fluoride, 30 mM sodium PP_i, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 20 nM microcystin, and 1% Triton X-100. After removal of insoluble material by centrifugation, cell extracts were pooled and aliquoted. The extracts were rotated with 0.2 μl of HA.11 ascites fluid and a secondary rabbit anti-mouse antibody bound to protein A-Sepharose beads. Immunoprecipitates were washed three times in wash buffer [50 mM HEPES (pH 7.6), 1 M sodium chloride, 10% glycerol, and 1% Triton X-100] and three times in kinase base buffer [50 mM Tris (pH 7.4), 10 mM $MgCl_2$]. Drug incubations were performed as described above. The kinase reaction mix was then added to yield final concentrations of 50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 10 μM [γ-^{32}P]ATP (specific activity, 50 Ci/mmol; ICN), and 25 ng/μl recombinant GST-$Cdc25C^{200-256}$ in a total volume of 40 μl. The phosphorylation reactions were incubated for 15 min at 30°C, and the incorporation of radioactive phosphate into GST-$Cdc25C^{200-256}$ was determined as described above.

p53 Phosphorylation. Subconfluent cultures of A549 cells were treated with 50 μM LLnL and the indicated concentrations of caffeine or wortmannin for 15 min prior to γ-irradiation. Cells were exposed to 0 or 20 Gy from a ^{137}Cs source at a dose rate of 6.4 Gy/min and then returned to 37°C. After 3 h, the cells were washed with PBS (pH 7.4) and lysed on ice in TNE buffer [50 mM Tris (pH 8.0), 150 mM sodium chloride, 5 mM EDTA, 1% NP40, 0.1% SDS, 1 mM DTT, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 20 nM microcystin, and 10 mM β-glycerophosphate]. Insoluble material was cleared by centrifugation, and soluble proteins (40 μg per sample lane) were resolved by SDS-PAGE. The levels of Ser^{15}-phosphorylated and nonphosphorylated p53 were determined as described (11).

A similar protocol was used to examine p53 phosphorylation following UV irradiation. A549 cells were incubated with 50 μM LLnL for 2 h before addition of the indicated concentrations of caffeine or wortmannin. After an additional 15 min, the culture medium was removed and replaced with HBSS (pH 7.4). The cells were exposed immediately to 0 or 50 $J \cdot m^{-2}$ UV radiation, and then the HBSS was replaced with fresh medium. The starting concentrations of LLnL, caffeine, and wortmannin were maintained throughout these manipulations by re-addition of the appropriate compounds following each change of the incubation medium. After 4 h, the cells were lysed and processed as described above.

Mitogen-activated Protein Kinase Phosphorylation. Subconfluent cultures of A549 cells were serum starved for 16 h and then were pretreated for 45 min with the MEK inhibitor, PD098059, or for 15 min with caffeine. The pretreated cells were stimulated with 100 ng per ml PMA for 5 min and then were lysed in TNE buffer. The detergent-soluble proteins were processed for

immunoblotting with phospho-Erk-specific antibodies. The protein blots were simultaneously probed with PLC-γ1-specific antibodies to insure that equal amounts of extract protein were loaded in each sample lane.

RDS. Inhibition of DNA synthesis after γ-irradiation of A549 cells was measured using a modification of thymidine incorporation assays described previously (28, 34). Subconfluent A549 cells were plated into 96-well plates (2000 cells per 100 μl/well), and the intracellular DNA pool was prelabeled with 1.5 nCi/well [*methyl*-^{14}C]thymidine (specific activity, 59 mCi/mmol; ICN). After 72 h, the indicated concentrations of caffeine or wortmannin were added to the cells. After 15 min, cells were exposed to 0 or 30 Gy γ-radiation as described above. Twenty min after irradiation, the cells were pulsed for 40 min with 2 μCi/well [*methyl*-^{3}H]thymidine (specific activity, 5 Ci/mmol; Amersham). Cells were harvested by trypsinization, transferred onto glass filters, and lysed in distilled water. Filter-bound radioactivity was determined by liquid scintillation counting. The relative DNA synthesis rate for a given drug treatment after radiation was calculated using the equation $([^3H]/[^{14}C]_{30\ Gy} \div ([^3H]/[^{14}C]_{0\ Gy}$. Each treatment condition was tested in six replicate wells.

Flow Cytometry. Subconfluent cultures of A549 cells were treated with 5 μM BrdUrd and the indicated concentrations of caffeine 15 min prior to γ-irradiation. Cells were exposed to 0 or 5 Gy and then returned to 37°C. After 6 h, cells were trypsinized and fixed in 70% ice-cold ethanol/30% PBS. Cells were processed for flow cytometry essentially as described previously (35). Briefly, following RNaseA treatment, pepsin digestion, and acid denaturation, samples were serially incubated with a primary anti-BrdUrd antibody and then a secondary FITC-conjugated antibody. Samples were resuspended in 20 μg/ml propidium iodide prior to analysis.

Simultaneous measurements of DNA content (red fluorescence) and BrdUrd content (green fluorescence) were obtained on all samples using a FACScan flow cytometer (Becton Dickinson). A minimum of 20,000 ungated events was recorded for each sample. Analyses of the data were performed using the WinMDI Version 2.7 software program.[5] Doublets and clumps were excluded from the analysis by gating on the DNA pulse width *versus* area. The percentage of BrdUrd-negative cells remaining in G_2 following DNA damage was determined by gating on the bivariate distribution of green height (BrdUrd-FITC) *versus* red area (propidium iodide; see Fig. 3*B*, *inset*).

Statistics. All statistical analyses were performed with the Sigma Plot 4.0 (SPSS) software package. The kinase inhibition data were fit with a linear-regression model by the least-squares method. The concentrations of caffeine resulting in half-maximal inhibition (IC_{50}) for each kinase were calculated by solving the respective linear models for a relative activity of 0.5. Equivalent IC_{50} values were obtained using a Hill four-parameter regression model.

RESULTS

Inhibition of PIKKs and hChk1 Kinase Activities by Caffeine. Several methylxanthine-derived drugs, including caffeine, theophylline, and pentoxifylline, have been shown to sensitize cells to radiation at low millimolar concentrations (24–26, 36). Although the exact mechanism remains unclear, the radiosensitizing effects of these drugs appear related to the inhibition of one or more components of the DNA damage-responsive, cell cycle checkpoint machinery. Because methylxanthines are purine analogues, we surmised that these compounds might inhibit the phosphotransferase activities of a protein kinase required for checkpoint signaling in DNA-damaged cells. Two prominent candidates for the protein kinase targeted by methylxanthines were ATM and hChk1. As an initial test of the sensitivities of the ATM and hChk1 kinase activities to caffeine and pentoxifylline, we performed immune complex kinase assays in the absence or presence of each drug. Treatment of ATM immunoprecipitates with 1 mM caffeine or 1 mM pentoxifylline strongly inhibited the phosphorylation of the exogenous substrate, PHAS-I (Fig. 1*A*). In parallel assays, we examined the sensitivity of ATM kinase activity to a second radiosensitizing agent, UCN-01. Interestingly, the protein kinase activity of immunoprecipitated ATM was not inhibited by 1 μM UCN-01 (Fig. 1*A*), despite the fact that this concentration of UCN-01 maximally sensitizes intact cells to ionizing radiation (23).

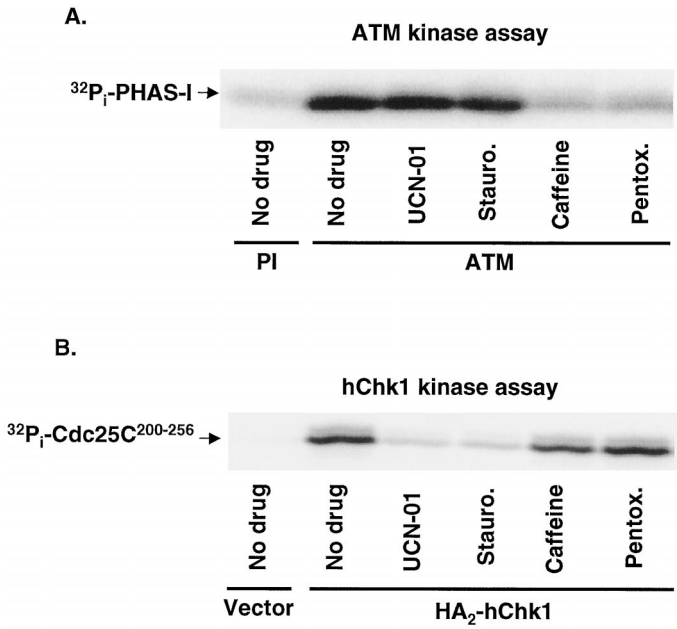

Fig. 1. Inhibition of ATM and hChk1 activities by radiosensitizing agents. *A*, preimmune (*PI*) or ATM immunoprecipitates from A549 cells were preincubated on ice with buffer alone or 2× final concentrations of the indicated checkpoint inhibitors diluted in kinase buffer. Kinase reactions were initiated with the addition of an equal volume of kinase buffer containing PHAS-I, $MnCl_2$, and [γ-^{32}P]ATP. The final concentrations of the inhibitors in the kinase reactions were 1 μM UCN-01, 1 μM staurosporine, 1 mM caffeine, and 1 mM pentoxifylline. The reaction products were resolved by SDS-PAGE, and incorporation of $^{32}P_i$ into PHAS-I was quantitated with a Molecular Dynamics Storm Phosphorimaging system and ImageQuant software. *B*, K562 erythroblastoid leukemia cells were transfected with empty vector or with pEF-BOS-hChk1-HA_2. Cells were lysed after 18–20 h, and hChk1 was immunoprecipitated with an HA-specific monoclonal antibody. The immunoprecipitates were preincubated on ice with the indicated compounds as described above. Kinase reactions were initiated by addition of [γ-^{32}P]ATP and the GST-Cdc25C$^{200-256}$ kinase substrate. The reaction products were resolved by SDS-PAGE, and the incorporation of $^{32}P_i$ into GST-Cdc25C$^{200-256}$ was determined by phosphorimaging. *Stauro.*, staurosporine; *Pentox.*, pentoxyfylline.

The sensitivity of the hChk1 kinase to methylxanthines and UCN-01 differed dramatically from that observed with ATM as the target kinase. Epitope-tagged hChk1 was immunoprecipitated from transiently transfected K562 cells, and immune complex kinase assays were performed with a GST-Cdc25C fusion protein as the substrate. The Cdc25C fragment contained the Ser^{216} residue that has recently been identified as a phosphorylation site for hChk1 (15, 16). In contrast to the results obtained with ATM immunoprecipitates, hChk1 kinase activity was minimally inhibited by 1 mM caffeine or pentoxifylline but was completely suppressed by 1 μM UCN-01 (Fig. 1*B*).

ATM is a member of a family of proteins that contain a PI3K-like catalytic domain (5). To determine whether caffeine exerted differential inhibitory effects on the protein kinase activities of the mammalian PIKK family members, we developed caffeine concentration-inhibition curves for ATM, ATR, mTOR, and DNA-PK (Fig. 2*A–D*), as well as hChk1 (Fig. 2*E*). The PHAS-I-phosphorylating activities of ATM, mTOR, and ATR exhibited similar sensitivities to caffeine, with 50% inhibition of kinase activity (IC_{50}) observed at drug concentrations of 0.2, 0.4, and 1.1 mM, respectively. The inhibitory potency of caffeine toward ATM was identical when a GST-p53 fusion protein was used as the substrate (data not shown). The latter substrate is phosphorylated at the Ser^{15} residue, which appears to be a physiological site for modification by ATM in DNA-damaged cells (12–14). On the other hand, the protein kinase activity of the DNA-PK heterotrimer was relatively resistant to caffeine (IC_{50}, 10 mM),

[5] J. Trotter, http://facs.scripps.edu.

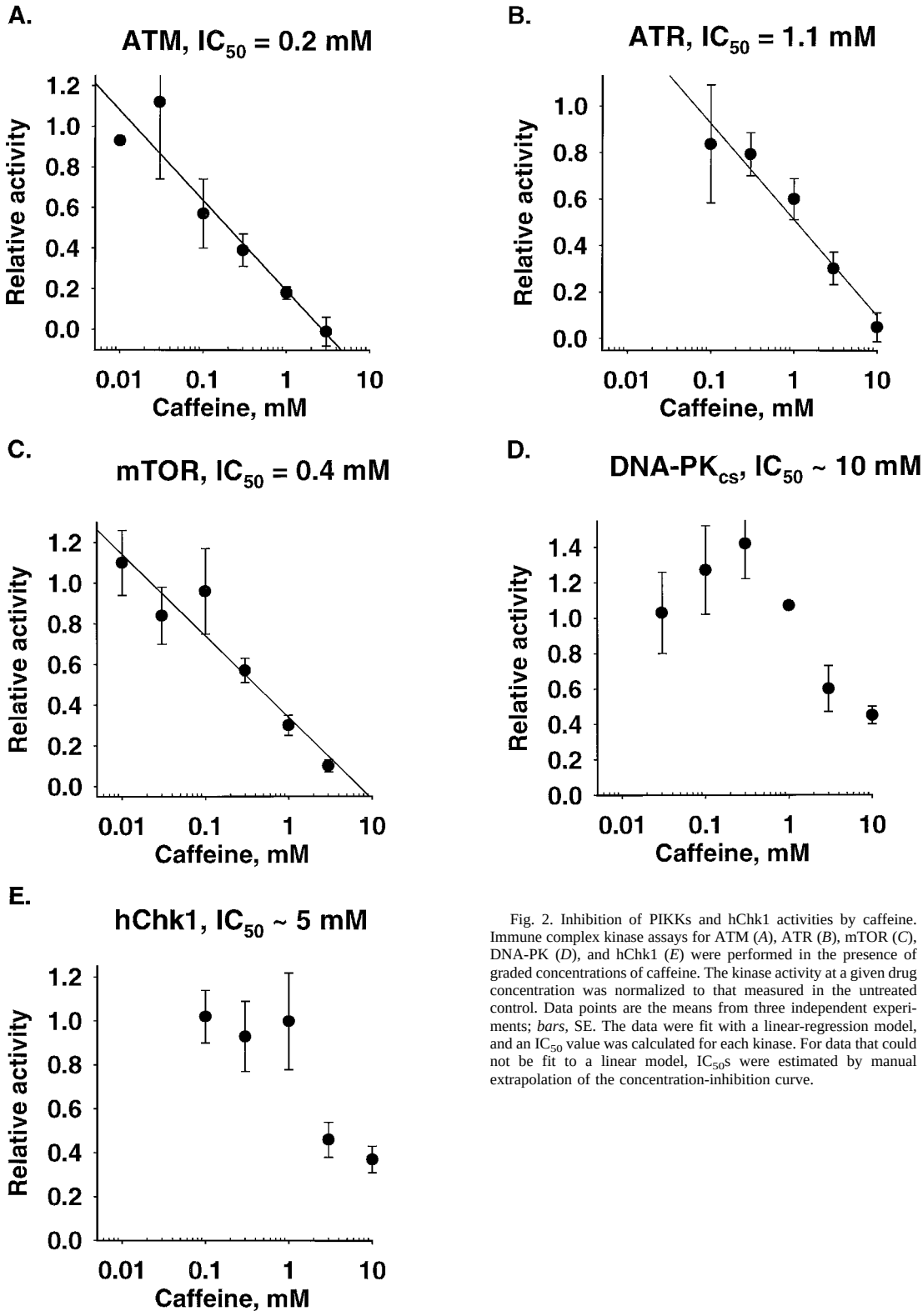

Fig. 2. Inhibition of PIKKs and hChk1 activities by caffeine. Immune complex kinase assays for ATM (*A*), ATR (*B*), mTOR (*C*), DNA-PK (*D*), and hChk1 (*E*) were performed in the presence of graded concentrations of caffeine. The kinase activity at a given drug concentration was normalized to that measured in the untreated control. Data points are the means from three independent experiments; *bars*, SE. The data were fit with a linear-regression model, and an IC_{50} value was calculated for each kinase. For data that could not be fit to a linear model, IC_{50}s were estimated by manual extrapolation of the concentration-inhibition curve.

whereas hChk1 displayed an intermediate level of sensitivity (IC_{50}, 5 mM). Because caffeine induces radiosensitization and inhibits checkpoint functions at concentrations of 0.5–2 mM, the results of our *in vitro* kinase assays suggest that DNA-PK and hChk1 are not relevant targets for the radiosensitizing effects of caffeine in intact cells (24–26, 36). Furthermore, other studies have shown that rapamycin, which is a highly selective inhibitor of mTOR kinase activity, does not sensitize cells to killing by ionizing radiation (37). Thus, these observations directed our attention toward the possible roles of ATM and ATR inhibition in the disruption of checkpoint functions by caffeine.

Effects of Caffeine on PIKK-dependent Checkpoint Functions. A hallmark abnormality of ATM-deficient cells is RDS, which presumably reflects the defective operation of a DNA damage-induced S phase checkpoint (28). If caffeine treatment actually inhibits ATM

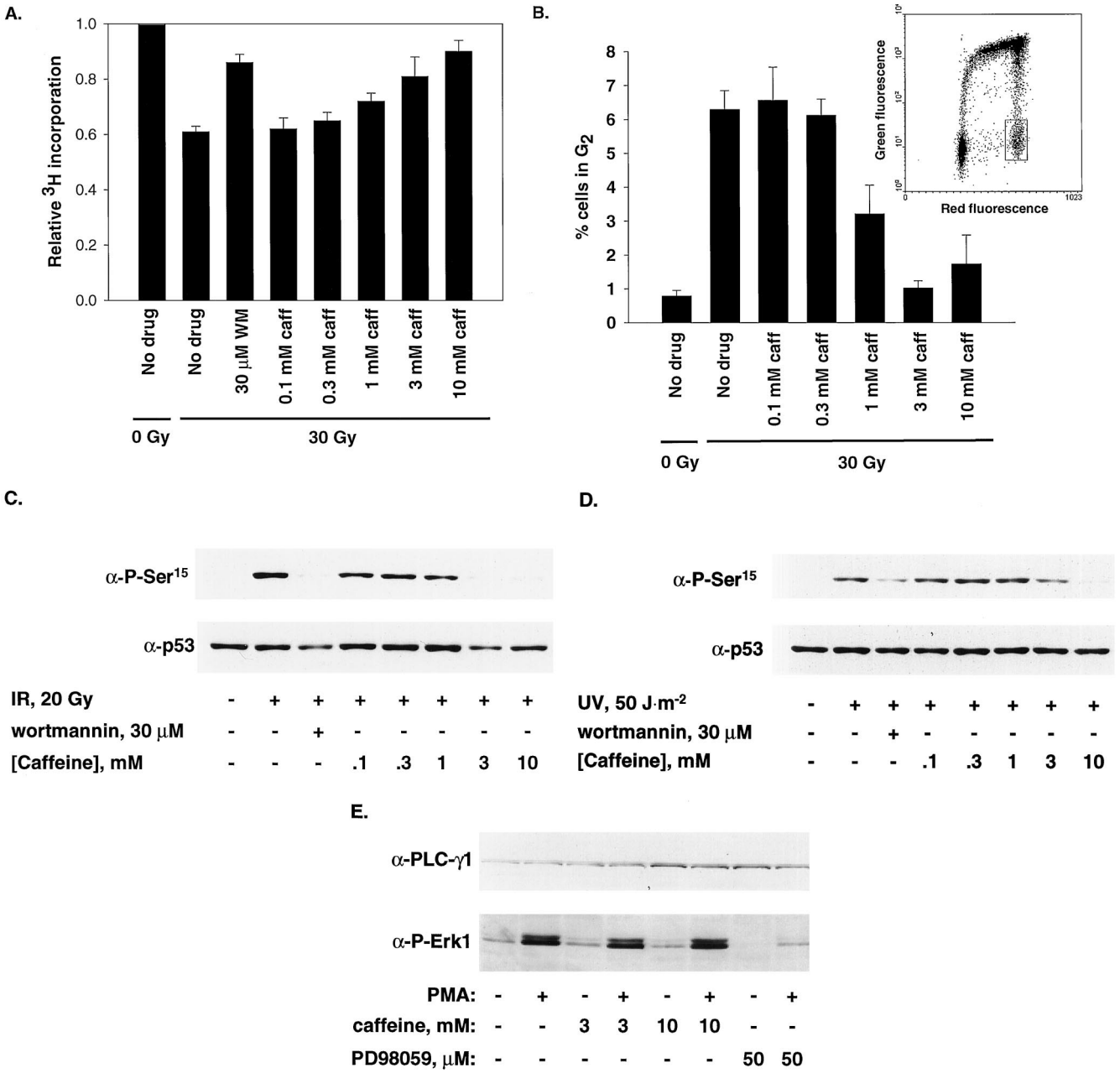

Fig. 3. Effects of caffeine on ATM and ATR function in intact cells. *A*, RDS. A549 cells were incubated with [*methyl*-^{14}C]thymidine for 72 h to label cellular DNA. The cells were then treated with wortmannin (*WM*) or graded concentrations of caffeine (*caff*), followed by exposure to 0 or 30 Gy γ-radiation. The irradiated cells were pulse-labeled for 40 min with [*methyl*-^{3}H]thymidine. Incorporation of radioactivity into DNA was assayed by liquid scintillation counting. For any given drug treatment, the ratio of ^{3}H *versus* ^{14}C incorporation in the irradiated sample was normalized to that obtained in the corresponding drug-treated, nonirradiated control. Results are presented as means of three independent trials; *bars*, SE. *B*, abrogation of the G_2-checkpoint. Graded concentrations of caffeine and 5 μM BrdUrd were added to the culture media of A549 cells 15 min before exposure to 5 Gy γ-irradiation. Cells were harvested 6 h later for FACS analysis. Fixed cells were stained with propidium iodide and an α-BrdUrd primary antibody/FITC-conjugated secondary antibody. The percentage of BrdUrd-negative cells arrested in G_2 after irradiation was determined by analysis of the bivariate plots of red fluorescence (DNA content) *versus* green fluorescence (BrdUrd content) for the various treatments (*inset*). The results are summarized graphically, and data are presented as the means of four independent trials; *bars*, SE. *Inset*, representative bivariate plot for cells treated with 5 Gy and 0 mM caffeine. *C*, γ-radiation-induced phosphorylation of p53. A549 cells were treated with a proteosome inhibitor (50 μM LLnL) and the indicated compounds for 15 min prior to γ-irradiation with 0 or 20 Gy. Cells were lysed after 3 h, and soluble proteins (40 μg in each sample lane) were resolved by SDS-PAGE. The proteins were blotted onto a polyvinylidene difluoride membrane and then were serially probed with anti-phospho-Ser^{15}-specific and anti-p53-specific antibodies. The results shown are representative of those obtained in three independent trials. Similar results were obtained when the phosphorylation of p53 on Ser^{15} was analyzed 30 min after irradiation (data not shown). *D*, UV light-induced p53 phosphorylation. A549 cells were incubated with 50 μM LLnL and the indicated compounds prior to exposure to 50 J·m^{-2} UV radiation. Cells were lysed after 4 h, and samples were processed for immunoblotting as described. *E*, Erk1 phosphorylation. Serum-starved A549 cells were incubated with caffeine or the MEK inhibitor, PD098059, prior to stimulation with 100 ng/ml phorbol ester (*PMA*). Cells were lysed 5 min later, and equal amounts of soluble protein were resolved by SDS-PAGE. Phosphorylation of Erk1 was detected by immunoblotting with phospho-specific Erk1 antibodies. The equivalency of protein loading in each sample lane was confirmed by blotting with PLC-γ1-specific antibodies.

kinase activity in intact cells, then these cells should also display the defective RDS phenotype. This prediction was supported by the finding that RDS was induced in A549 cells by treatment with concentrations of caffeine that correspond closely with those required for significant inhibition of ATM kinase activity (Fig. 3*A*). As reported previously, A549 cells also displayed the RDS phenotype after treatment with the structurally distinct ATM inhibitor, wortmannin (34). The observed differences in DNA synthesis inhibition for the

irradiated cells pretreated with ATM-inhibitory concentrations of caffeine or wortmannin, compared with no drug treatment, are similar to the studies reported previously of A-T *versus* wild-type fibroblasts (28).

A second checkpoint defect characteristic of ATM-deficient cells is the failure of cells irradiated in the G_2 phase of the cell cycle to arrest prior to mitosis (38). As with the S-phase checkpoint, we hypothesized that caffeine should abrogate this G_2 checkpoint at concentrations associated with significant inhibition of intracellular ATM kinase activity. To examine the fate of cells irradiated in the G_2 phase of the cell cycle, BrdUrd was added to the incubation medium just prior to drug treatment and irradiation. BrdUrd is a thymidine analogue that is incorporated into DNA during replication and can be recognized by a specific α-BrdUrd antibody. The cell cycle distribution of a mixed population of BrdUrd-labeled and -unlabeled cells can be assessed using a bivariate flow cytometric analysis of DNA content *versus* BrdUrd intensity. Therefore, at time points following BrdUrd addition and irradiation, cells arrested in G_2-M (*i.e.,* those with 4N DNA content), which do not contain BrdUrd, must have been in G_2-M at the time of irradiation. (Fig. 3*B, inset*). Using this technique, A549 cells irradiated in G_2-M remained arrested in that phase of the cell cycle 6 h later. However, pretreatment of these cells with caffeine at concentrations $>$0.3 mM significantly abrogated this checkpoint (Fig. 3*B*). The dose-response relationship for abrogation of this ATM-dependent G_2-checkpoint response correlates closely with the range of caffeine concentrations associated with significant radiosensitization (25, 26).

Recent studies have suggested that ATM and ATR are directly responsible for the phosphorylation of the NH_2 terminus of p53 at Ser^{15} after cellular exposure to γ-radiation or UV light (11–14). Furthermore, these studies provide indirect evidence that ATM plays a major role in the early phase of Ser^{15} phosphorylation in γ-irradiated cells, whereas ATR may be more important for UV light-induced phosphorylation of this site in p53. On the basis of the premise that caffeine treatment inhibits the protein kinase activities of both ATM and ATR, we predicted that the γ- and UV radiation-inducible phosphorylation of Ser^{15} would be blocked by caffeine. The intensity of the Ser^{15} phosphorylation response in the irradiated cells was monitored by immunoblotting of cellular extracts with phospho-Ser^{15}-specific (α-P-Ser^{15}) antibodies (33). In these experiments, the cells were pretreated with the proteosome inhibitor, LLnL, to block the degradation of p53 in irradiated and unirradiated cells. LLnL treatment stabilizes the total cellular levels of p53, thereby facilitating the interpretation of changes in α-P-Ser^{15} immunoreactivity after cellular exposure to DNA-damaging agents (33, 39). Caffeine treatment strongly inhibited γ-radiation- and UV light-inducible phosphorylation of p53 at Ser^{15} (Fig. 3, *C* and *D*), at concentrations similar to those required for significant inhibition of ATM and ATR kinase activities, respectively. The phosphorylation of Ser^{15} in γ-irradiated cells was also suppressed by pretreatment of the cells with 30 μM wortmannin, a drug concentration known to inhibit the kinase activity of ATM, but not ATR, in intact cells (34). Interestingly, wortmannin treatment also inhibited Ser^{15} phosphorylation of p53 after UV radiation, which suggests that ATM or another wortmannin-sensitive protein kinase participates in UV light-induced p53 modification in A549 cells.

As a purine analogue, caffeine might interfere with checkpoint signaling functions simply by acting as a broad-spectrum kinase inhibitor. Although the relative resistance of hChk1 and DNA-PK activity to caffeine argues against this possibility, we wished to address this issue further by examining the effect of caffeine on the function of a well-studied cytoplasmic signaling cascade, the Ras to Erk pathway (40). Serum-starved A549 cells were stimulated with the phorbol ester PMA, and activation of Erk1 was determined with phospho-Erk1-specific antibodies (Fig. 3*E*). As expected, the Erk1 activation response was strongly suppressed by pretreatment of the cells with PD098059, an inhibitor of MEKs, the upstream activators of Erks. In contrast, caffeine pretreatment had no effect on PMA-induced Erk1 activation in A549 cells (Fig. 3*E*), indicating that concentrations of caffeine that interfere with checkpoint signaling functions do not cause a global inhibition of conventional protein kinase activities in these cells.

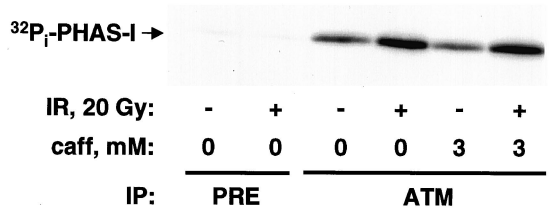

Fig. 4. Effects of caffeine on γ-radiation-induced ATM activation. A549 cells were treated with 0 or 3 mM caffeine (*caff*) prior to exposure to 0 or 20 Gy. Cells were lysed after 60 min, and ATM kinase activity was determined with PHAS-I as the substrate. *IP,* immunoprecipitation.

Effect of Caffeine on Radiation-induced ATM Activation. The results presented to this point support the hypothesis that the radiosensitizing effects of caffeine are related to the inhibition of ATM and ATR kinase activities. However, because cellular exposure to γ-radiation increases the *in vitro* kinase activity of ATM (13, 14), a caffeine-induced block in an upstream event leading to ATM activation might also confer multiple checkpoint defects in drug-exposed cells. To examine this possibility, A549 cells were pretreated with 3 mM caffeine and then irradiated with 20 Gy γ-radiation. Cells were then lysed, and the kinase activity of immunoprecipitated ATM was assessed. Because caffeine presumably functions as a reversible inhibitor of ATM kinase activity, we expected to recover catalytically active ATM when caffeine was removed during the process of immunoprecipitation. In fact, the protein kinase activity of ATM was increased by ~2-fold after cellular irradiation, and this increase was not affected by pretreatment of the cells with caffeine (Fig. 4). Thus, in contrast to the protein kinase activity of ATM itself, the pathway leading to ATM activation in irradiated cells is not sensitive to caffeine. Moreover, these results suggest that the mechanism of ATM activation by γ-radiation does not involve either auto- or *trans*-phosphorylation of ATM by either of the caffeine-sensitive PIKKs, ATM or ATR.

DISCUSSION

Although the radiosensitizing properties of caffeine have been studied for over two decades, the mechanism underlying this drug effect had not been identified. The abilities of caffeine and related methylxanthines to block radiation-induced G_2 arrest suggested that these drugs might target one or more of the proteins involved in activation of the G_2 checkpoint. In this report, we provide pharmacological and biochemical evidence that caffeine and the structurally related methylxanthine, pentoxifylline, exert their checkpoint-abrogating activities by inhibiting the protein kinase activities of ATM and ATR. These proteins are members of the PIKK family, and genetic results have strongly implicated ATM and ATR as proximal components of DNA damage-responsive checkpoints, including the G_2 checkpoint, in mammalian cells. Moreover, on the basis of the profound radiation sensitivity of ATM-deficient cells or cells overexpressing kinase-dead ATR, we suggest that the inhibition of ATM and ATR by caffeine is responsible for the radiosensitizing effects of this compound (7, 8, 41). In contrast, a second known inhibitor of the G_2

checkpoint, the staurosporine analogue, UCN-01, did not inhibit ATM at concentrations known to induce radiosensitization and G_2 checkpoint abrogation. Instead, this compound proved to be a potent inhibitor of the hChk1 kinase, an important downstream signal transducer in the checkpoint pathway leading to Cdc25C inhibition in DNA-damaged cells.

In previous studies, we demonstrated that the protein kinase activities of mTOR, ATM, and DNA-PK were inhibited by wortmannin (IC_{50}s, 16–250 nM), whereas ATR activity was at least 10-fold more resistant to this drug (32, 34). On the basis of the observation that the specific mTOR inhibitor, rapamycin, does not confer a radiosensitive phenotype (37), then ATM and DNA-PK emerge as the candidate molecular targets for wortmannin-mediated radiosensitization. In the present studies, we observed that caffeine, although not a global inhibitor of conventional protein kinase activities (Fig. 3*E*), effectively inhibited the phosphorylation of protein substrates by all four mammalian PIKKs. In contrast to the results obtained with wortmannin, the catalytic activity of DNA-PK was the most resistant to inhibition by caffeine. On the basis of the *in vitro* potency data for ATR, ATM, and DNA-PK, we surmise that inhibition of ATR and ATM correlates most closely with radiosensitization and the G_2 checkpoint bypass observed in cells treated with low millimolar concentrations of caffeine. This conclusion is supported by the earlier finding that the intracellular concentration of caffeine rapidly equilibrates with that present in the extracellular medium (42). Hence, the potencies of caffeine as an inhibitor of ATM and ATR kinase activities in intact cells should approximate those obtained in the immune complex kinase assays. Although the contribution of other protein targets cannot be excluded, our results suggest that ATM is a common target for both wortmannin- and caffeine-mediated radiosensitization, whereas the concomitant inhibition of ATR activity by caffeine may contribute to the distinct radiosensitizing properties of this drug.

The disruptive effects of caffeine on the G_1 and S-phase checkpoints are consistent with a drug-induced loss of ATM function in treated cells. A central regulator of the G_1 checkpoint is p53, and studies in ATM-deficient cells indicate that ATM is required for the timely phosphorylation and activation of p53 after cellular exposure to ionizing radiation or radiomimetic agents (12). Indeed, ATM may be directly responsible for the phosphorylation of p53 at Ser^{15} in γ-irradiated cells (13, 14). Our results show that caffeine treatment blocks γ-radiation-induced Ser^{15} phosphorylation at drug concentrations that inhibit the *in vitro* protein kinase activity of ATM by >80%. The idea that caffeine confers an A-T-like phenotype on ATM-positive cells is further substantiated by the observation that similar concentrations of caffeine abrogate S- and G_2-phase cell cycle checkpoints. Loss of these checkpoints is a characteristic abnormality of cells derived from A-T patients. Interestingly, we have reported that treatment of A549 cells with ATM-inhibitory concentrations of wortmannin also abrogates the S-phase checkpoint, which results in an RDS phenotype (34). It is conceivable that the induction of this RDS phenotype might form the basis for a convenient cell-based screen for additional small molecule inhibitors of the S-phase checkpoint pathway governed by ATM.

The spectrum of checkpoint defects induced by caffeine treatment suggests that ATM is not the only checkpoint component affected by this drug:

(*a*) ATM-deficient cells display a normal level of sensitivity to UV radiation, whereas caffeine treatment renders cells hypersensitive to this DNA-damaging agent. Interestingly, recent results from our laboratory suggest that ATR, rather than ATM, may be the principle regulator of p53 activation after UV light exposure (11). Consistent with this observation, we now show that caffeine inhibits the phosphorylation of p53 on Ser^{15} after UV light exposure at concentrations associated with significant inhibition of ATR kinase activity *in vitro*. The inhibition of ATR-dependent checkpoint functions by caffeine is also consistent with the sensitization of p53-null cells by UV light (43). Collectively, these results suggest that ATR is a primary participant in both p53-dependent and -independent checkpoint pathways triggered by UV light exposure.

(*b*) γ-irradiation of A-T cells during G_1 or S phase results in a protracted arrest in G_2-phase. Caffeine shortens or eliminates the γ-radiation-induced G_2 arrest in ATM-positive cells and abrogates the prolonged G_2 arrest seen in γ-irradiated A-T fibroblasts (36, 44, 45). These results suggest that an ATM-independent pathway enforces the G_2 checkpoint in A-T cells and that this alternative pathway is disrupted by caffeine. We propose that this alternative checkpoint pathway is mediated through ATR, and that the abrogation of the radiation-induced G_2 arrest by caffeine reflects the concomitant inhibition of ATM and ATR kinase activities by this drug. In support of this notion, we observed that wortmannin-treated cells, like A-T cells, accumulate in G_2 phase after exposure to γ-radiation. This phenotype is consistent with the fact that wortmannin inhibits the protein kinase activity of ATM, but not ATR, at drug concentrations that induce maximal radiosensitization (34). These observations imply that caffeine inhibits not only ATM but at least one additional checkpoint protein involved in the control of the G_2 checkpoint and the cellular response to UV damage. Our pharmacological data suggest that this target may be ATR.

An important issue with respect to ATM and ATR inhibitors as potential chemotherapeutic agents is whether this class of drugs will show selectivity for tumor *versus* normal tissue. A frequent distinction between normal cells and cancer cells is that the latter have lost p53-dependent checkpoint controls, often through mutation of p53 itself (46). The loss of p53 function abrogates the G_1 checkpoint and may compromise a G_2 checkpoint pathway that operates in parallel with those governed by ATR and/or ATM (47–51). Recent evidence suggests that the therapeutic efficacies of certain DNA-damaging anticancer drugs are causally related to the loss of normal DNA damage checkpoint controls during the process of carcinogenesis (52, 53). These studies suggest that agents that interfere with checkpoint-related proteins may show selectivity for tumor cells bearing intrinsic defects in specific checkpoint pathways. Indeed, this prediction is supported by the observation that p53-deficient cells are preferentially sensitized to radiation-induced killing by caffeine (24–26), which, based on the present findings, targets ATM and ATR. Similarly, the potent inhibition of Chk1 kinase activity by UCN-01 may explain the increased efficacy of this drug as a radiosensitizing agent in p53-mutated cells (23).

The notion that p53-null tumor cells are "marked" for sensitization by inhibitors of ATM, ATR, hChk1, or other checkpoint control proteins is clearly provocative from the viewpoint of cancer therapy. The results of the present studies suggest that the archetypal radiosensitizing agent, caffeine, inhibits the protein kinase activities of ATM and ATR at concentrations overlapping those that cause moderate radiosensitization in p53-null tumor cells. However, whether suppression of ATM and/or ATR kinase activities will actually increase the therapeutic indices of existing anticancer therapies in patients awaits the development of more potent and specific inhibitors of these PIKK family members.

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Inhibition of ATM and ATR Kinase Activities by the Radiosensitizing Agent, Caffeine

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