

MK-1775, a Novel Wee1 Kinase Inhibitor, Radiosensitizes p53-Defective Human Tumor Cells

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Abstract

Purpose: Radiotherapy is commonly used to treat a variety of solid tumors. However, improvements in the therapeutic ratio for several disease sites are sorely needed, leading us to assess molecularly targeted therapeutics as radiosensitizers. The aim of this study was to assess the wee1 kinase inhibitor, MK-1775, for its ability to radiosensitize human tumor cells.

Experimental Design: Human tumor cells derived from lung, breast, and prostate cancers were tested for radiosensitization by MK-1775 using clonogenic survival assays. Both p53 wild-type and p53-defective lines were included. The ability of MK-1775 to abrogate the radiation-induced G₂ block, thereby allowing cells harboring DNA lesions to prematurely progress into mitosis, was determined using flow cytometry and detection of γ -H2AX foci. The *in vivo* efficacy of the combination of MK-1775 and radiation was assessed by tumor growth delay experiments using a human lung cancer cell line growing as a xenograft tumor in nude mice.

Results: Clonogenic survival analyses indicated that nanomolar concentrations of MK-1775 radiosensitized p53-defective human lung, breast, and prostate cancer cells but not similar lines with wild-type p53. Consistent with its ability to radiosensitize, MK-1775 abrogated the radiation-induced G₂ block in p53-defective cells but not in p53 wild-type lines. MK-1775 also significantly enhanced the antitumor efficacy of radiation *in vivo* as shown in tumor growth delay studies, again for p53-defective tumors.

Conclusions: These results indicate that p53-defective human tumor cells are significantly radiosensitized by the potent and selective wee1 kinase inhibitor, MK-1775, in both the *in vitro* and *in vivo* settings. Taken together, our findings strongly support the clinical evaluation of MK-1775 in combination with radiation. *Clin Cancer Res*; 17(17); 5638–48. ©2011 AACR.

Introduction

Tumor cells typically have high rates of cell proliferation that render them especially sensitive to DNA-damaging agents, which include several classes of conventional cancer chemotherapy drugs and ionizing radiation, and these agents continue to have important roles in therapeutic treatments. Unfortunately, in some critical normal tissues, homeostasis is maintained by the proliferation of their

stem cell compartments, and the cytotoxic effects of these DNA-damaging agents on such stem cells severely limits their efficacy for certain cancers. This has prompted the development of a new approach to cancer treatment, referred to as molecularly targeted therapy, and several molecularly targeted drugs have been approved for clinical use in the past few years. Molecularly targeted therapy takes advantage of our increasing understanding of the molecular abnormalities associated with tumor cells that are responsible for their malignant phenotype, and these new drugs are designed to specifically target such molecules thereby offering specificity that is lacking for the conventional agents.

Of all of the molecular abnormalities associated with tumor cells, one of the most common, estimated to be present in 50% of all cancers, is loss of function of the tumor suppressor protein p53 (1). p53 is a transcription factor that plays a pivotal role in the cellular response to DNA-damaging agents by facilitating either cell-cycle arrest or cell death (2). Generally, cells arrest in the cell cycle following damage to their DNA at 3 well-established checkpoints which occur in G₁, S, and in G₂ phase (3). Arresting at these checkpoints allows the cells more time to

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi: 10.1158/1078-0432.CCR-11-0650

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Translational Relevance

There is increasing interest in combining molecularly targeted drugs with radiotherapy as a strategy for enhancing the therapeutic ratio and, thus, patient response. Considering that many, if not most, human tumors have defective p53 function, an attractive approach to this strategy is to use agents that abrogate the radiation-induced G₂ block as radiosensitizers. One such agent, MK-1775, a wee1 kinase inhibitor, is currently in phase I clinical trials as a chemosensitizer in combination with the DNA-damaging agents gemcitabine, carboplatin, and cisplatin. The present study shows that MK-1775 also potently radiosensitizes p53-defective human tumor cells *in vitro* and *in vivo*. If the degree of radiosensitization observed in these preclinical experiments could be recapitulated in patients undergoing fractionated radiotherapy, this strategy would be predicted to have significant benefit. Thus, these results strongly support the clinical development of MK-1775 in combination with radiation.

repair the DNA damage before initiating cell division at mitosis; cells that attempt to undergo mitosis while continuing to harbor DNA lesions will likely die by a combination of cell death processes including apoptosis, mitotic death, and senescence (4, 5). p53 is primarily responsible for mediating the G₁ checkpoint and, therefore, DNA-damaged cells that lack normal p53 function extensively rely on the G₂ checkpoint for survival (6–8). The concept emerges then that abrogation of the G₂ checkpoint might preferentially sensitize p53-defective tumor cells to DNA-damaging agents and spare normal cells with intact p53 function (9). Thus, developing novel molecularly targeted drugs that abrogate the G₂ checkpoint has become an active area of research. Several such drugs have already been developed and tested either as single agents or in combination with conventional, DNA-damaging cancer chemotherapy agents (9, 10). Considering the importance of the G₂ block to the survival of irradiated cells (11, 12), it is logical to extend this approach to radiotherapy.

Design of agents that abrogate the G₂ block depends on understanding the molecular mechanisms involved. In G₂ phase, subsequent entry into mitosis is mainly controlled by activation of the cdc2/cyclin B complex, that is, dephosphorylation of tyrosine 15 (Tyr15) of cdc2 by cdc25 activates this complex thereby allowing entry into mitosis (13, 14). Following DNA damage, the G₂ checkpoint is initiated when cdc2 is inactivated by its phosphorylation on Tyr15 by the wee1 tyrosine kinase (15). In this latter situation, wee1 is activated and cdc25 inactivated downstream of the ATM-Chk1 signaling pathway that recognizes lesions in the DNA and signals cell-cycle checkpoints (16). Abrogation of the G₂ block could, therefore, be achieved through inhibition of the tyrosine kinase activity of wee1. In fact, it has been reported previously that abrogation of

wee1 activity, either by decreasing its expression using siRNA (17) or by inhibiting its tyrosine kinase activity with a small molecule inhibitor (18), sensitizes tumor cells to DNA-damaging agents.

In 2009, Hirai and colleagues described a novel, small molecule inhibitor of wee1, MK-1775, that is both potent and selective (19). In their initial report, they showed that MK-1775 inhibits the phosphorylation of cdc2 on Tyr15 with an IC₅₀ of 5.2 nmol/L in *in vitro* kinase assays. In tests of activity using a panel of 223 kinases, MK-1775 displayed substantial selectivity for wee1, for example, MK-1775 was 100 times more selective for wee1 as for Myt1, another kinase that suppresses cdc2 activity via phosphorylation at threonine 14 (20). Furthermore, they showed that MK-1775 abrogated the G₂ checkpoint and potentiated the cytotoxicity of the DNA-damaging agents gemcitabine, carboplatin, and cisplatin selectively in p53-deficient cells. In a subsequent report, this group showed that MK-1775 enhances the antitumor effects of additional DNA-damaging agents with different modes of action including 5-fluorouracil (5-FU), capecitabine, camptothecin, mitomycin C, doxorubicin, and pemetrexed (21). In light of the findings from these preclinical studies, MK-1775 has entered phase I clinical trials as a chemosensitizer in combination with gemcitabine, carboplatin, or cisplatin in patients with advanced solid tumors (22).

The purpose of the present study was to investigate the ability of MK-1775 to radiosensitize human tumor cells *in vitro* and *in vivo*. Our data indicate that MK-1775 preferentially radiosensitizes human tumor cells with defective p53 by abrogating the radiation-induced G₂ block thereby allowing irradiated cells harboring unrepaired DNA lesions to pass into mitosis and enter the next cell cycle. This radiosensitizing effect was consistent with mitotic death but not apoptosis. Tumor cells with wild-type p53 and cells of normal tissue origin were not similarly radiosensitized. Overall, these results suggest that MK-1775 may have clinical utility when combined with radiotherapy for the treatment of solid tumors with defective p53.

Materials and Methods

Cell cultures and reagents

The human cell lines A549, H1299, Calu-6, H460, CCD-16, MCF-7, MDA-MB-231, MCF-10A, PC3, and LNCaP were all obtained from the American Type Culture Collection (ATCC) and routinely maintained in RPMI-1640 medium supplemented with 10% FBS, 10,000 U/mL of penicillin-streptomycin, and 2 mmol/L glutamine. The identities of these cell lines were validated during the course of this study by short tandem repeat (STR) profiling conducted by the Institution's Characterized Cell Line Core using the AmpFI STR Identifier PCR Amplification Kit according to the manufacturer's instructions (Applied Biosystems). The STR profiles for these cell lines matched their known ATCC fingerprints. The H1299 cells with ponesterone A (Pon A)-inducible p53 expression have been described previously (23) and were the kind gift of

Dr. Jack Roth, Department of Thoracic Surgery, MD Anderson Cancer Center. MK-1775 was provided by Merck Sharp & Dohme Corp., and its chemical structure has been described previously (19). Cells were trapped in mitosis using 0.2 $\mu\text{g}/\text{mL}$ of nocodazole (Sigma-Aldrich).

Antibodies

Antibodies to cdc2 (#9112), p-cdc2 (Tyr15; #9111s), β -actin (#4967L), and phospho-Histone H3 (p-HH3, #9706) were purchased from Cell Signaling Technology. Antibodies to p53 (sc-263) were purchased from Santa Cruz Biotechnology and γ -H2AX (Ser139) clone JBW301 (05-636) antibody was purchased from Millipore.

Western blot analysis

Total protein was extracted from the cell pellet using a lysis solution containing 50 mmol/L HEPES (pH = 7.9), 0.4 mol/L NaCl, and 1 mmol/L EDTA and fortified with 10 $\mu\text{L}/\text{mL}$ phosphatase inhibitor cocktail 1, 10 $\mu\text{L}/\text{mL}$ phosphatase inhibitor cocktail 2, 10 $\mu\text{L}/\text{mL}$ protease inhibitor purchased from Sigma-Aldrich, and 1% NP-40. Protein concentration of the lysates was determined by the Bio-Rad protein assay. Equal amounts of protein were separated by 12% SDS-PAGE and transferred to an Immobilon membrane (Millipore). Nonspecific-binding sites on the membrane were blocked in 5% nonfat dry milk in Tris (20 mmol/L)-buffered saline (150 mmol/L, pH = 7.4) with 0.1% Tween (TBS with Tween). Protein signals were detected by incubating the membrane in primary antibody in 5% nonfat dry milk overnight at 4°C, followed by a 45-minute incubation in the appropriate peroxidase-conjugated secondary antibody. The membrane was then developed by enhanced chemiluminescence with ECL plus Western Blotting Detection Reagents (Amersham) on a Typhoon 9400 scanner (GE Healthcare).

Clonogenic assay

The effectiveness of the combination of MK-1775 and ionizing radiation was assessed by clonogenic assays. Briefly, cells growing in log phase were treated with 200 nmol/L MK-1775 1 hour prior to irradiation. Following irradiation, the cells were subjected to an 18-hour post-irradiation treatment with 200 nmol/L MK-1775. The cells were then trypsinized and counted, and known numbers were seeded in 60-mm culture dishes in 2 sets of 3 for each dose of radiation. Sufficient numbers were seeded to ensure that about 30 to 100 macroscopic colonies would appear in each plate after 10 to 14 days. Colonies were stained with 0.5% gentian violet in methanol and counted. The plating efficiency (PE) for each dose was calculated by dividing the number of colonies by the number of cells plated and expressing the result as a percentage. The surviving fraction was calculated by dividing the PE of the treatment by the PE of the appropriate unirradiated control.

Cell-cycle analysis

Cells were treated for 1 hour with 200 nmol/L MK-1775, irradiated at 7.5 Gy, and then harvested at 0, 4, 8, 12, 16,

and 24 hours later. The cells were then washed with PBS and fixed in 70% ethanol in PBS overnight at 4°C. The fixed cells were washed in buffer A [0.5% bovine serum albumin (BSA) and 2% FBS in PBS] and then incubated in lysis buffer (0.1% Triton X-100, 0.5% BSA, and 2% FBS in PBS) on ice for 5 minutes. The cells were pelleted by centrifugation and incubated in buffer B (2% BSA and 10% FBS in PBS). Again, cells were pelleted by centrifugation and then incubated with p-HH3 antibody at a dilution of 1:50 in buffer A overnight at 4°C. The cells were then washed with buffer A at room temperature and incubated for 1 hour in anti-mouse fluorescein isothiocyanate (FITC) secondary antibody at a dilution of 1:100 in buffer A. Cells were again washed with buffer A, pelleted by centrifugation, and incubated in 2% BSA, 2% Tween-20, 5 $\mu\text{g}/\text{mL}$ propidium iodide (PI; Sigma-Aldrich), and 2 $\mu\text{g}/\text{mL}$ RNase A (Sigma-Aldrich) for 1 hour in the dark, and flow cytometric analysis was conducted immediately thereafter. Flow cytometry was conducted using a Beckman Coulter EPICS-ALTRA with Hypersort system equipped with a water-cooled Argon laser emitting at 488 nm. Analysis was conducted using EXPO32 software. p-HH3 was measured using a 525-nm band pass filter. A minimum of 10,000 events were collected for analysis. Gates were set to exclude cellular debris, and the fluorescence intensity of events within the gated region was measured.

Immunofluorescence

A549 or H1299 cells were cultivated on coverslips placed in 35-mm dishes and treated with 0.2 $\mu\text{g}/\text{mL}$ nocodazole, irradiated with 1 Gy, and treated with 200 nmol/L MK-1775 as indicated. The medium was then aspirated, and the cells were rinsed briefly in PBS and then fixed with 2% paraformaldehyde for 15 minutes. Permeabilization was achieved by a 10-minute incubation with 100% methanol at -20°C . After three 5-minute rinses in PBS, the cells were incubated in blocking buffer (1 \times PBS, 50 $\mu\text{L}/\text{mL}$ normal goat serum, and 0.3% Triton X-100) for 1 hour at room temperature. Next, the cells were incubated in γ -H2AX (Millipore) primary antibody in antibody dilution buffer (1 \times PBS, 10 mg/mL BSA, 0.3% Triton X-100) overnight at 4°C with gentle shaking. After being washed with PBS, primary antibodies were visualized after a 2-hour incubation with the appropriate Alexa Fluor-conjugated secondary antibody (goat anti-rabbit FITC or goat anti-mouse Alexa Fluor 594) at a 1:500 dilution. Nuclei were counterstained with 1:500 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS, and the coverslips were mounted on slides with Vectashield (Vector Laboratories). Slides were examined using a Leica fluorescence microscope equipped with a CCD camera and images were imported into Advanced Spot Image analysis software. To quantify γ -H2AX foci, 50 nuclei were evaluated. Micronucleated cells were identified by DAPI staining and quantified (200 cells per coverslip).

Animals, tumor treatments, and tumor growth delay

Male NCr *nu/nu* mice were used for tumor studies. Animals were maintained in an Association for

the Assessment and Accreditation of Laboratory Animal Care approved facility in accordance with current regulations of the U.S. Department of Agriculture and Department of Health and Human Services. Experimental methods were approved by and in accordance with institutional guidelines established by the Institutional Animal Care and Use Committee.

Tumor xenografts were produced in the leg by intramuscular inoculation of 1×10^6 Calu-6 cells in 10 μ L. Irradiation and MK-1775 treatment were started when tumors reached 8-mm diameter and continued for 5 days. γ -rays were delivered locally to the tumor-bearing legs of unanesthetized mice using a small animal irradiator (Atomic Energy of Canada) consisting of 2 parallel-opposed ^{137}Cs sources, at a dose rate of 5 Gy/min. Tumors were irradiated twice daily separated by 6 hours. MK-1775 was given by gavage in 0.1 mL volumes 1 hour before and 2 hours after the first daily radiation dose.

Three mutually orthogonal tumor diameters were measured 2 to 3 times/wk with a Vernier caliper and means calculated for plotting tumor growth delay. Mice were euthanized when tumors grew to 15 mm diameter. Tumor regrowth was expressed as the time in days for tumors in the treated groups to grow from 8- to 12-mm diameter minus the time for control tumors to reach the same size [absolute growth delay AGD]. For treatment with both MK-1775 and radiation, normalized growth delay (NGD) was determined as time for tumors in the combined therapy group to grow to 12 mm minus time for tumors treated with drug alone to grow to 12 mm. Radiation enhancement factor (EF) was determined by dividing NGD for MK-1775 plus radiotherapy by the AGD for irradiation plus vehicle. *P* values for EFs were determined by Student's *t* test comparing NGD for MK-1775 plus irradiation versus AGD for irradiation plus vehicle. Differences were considered significant if $P \leq 0.05$.

Statistical analysis

Statistical significance was assessed by Student's *t* test (2 sample assuming unequal variances) and expressed as mean \pm standard error. A difference was considered significant if $P < 0.05$.

Results

MK-1775 radiosensitizes human tumor cells in a p53-dependent manner

We assessed the ability of MK-1775 to radiosensitize human tumor cells using clonogenic survival curve assays. Various cell lines were tested including lines derived from non-small-cell lung cancer (NSCLC), breast cancer, and prostate cancer. The p53 status of all of these lines had been previously established. In their original report on MK-1775, Hirai and colleagues showed that a concentration of 200 nmol/L of MK-1775 resulted in substantial suppression of p-cdc2 and that the optimal schedule was to first treat with the chemotherapy drug followed by a subsequent treatment with MK-1775 (19). The optimal concentration

of MK-1775 and sequence of administration relative to irradiation were validated in preliminary, pilot studies using the NSCLC lines A549 and H1299. We determined that 24-hour treatments with 200 nmol/L prior to irradiation were ineffective but 18-hour treatments after irradiation modestly radiosensitized p53-null H1299 cells. Subsequent experiments showed that concentrations higher than 200 nmol/L were too toxic for these cells. Furthermore, in a detailed test of additional sequences using H1299 cells, we found that the optimum sequence consisted of a 1-hour pretreatment followed by an 18-hour postirradiation treatment (Supplementary Fig. S1).

Using this treatment protocol, complete clonogenic survival curves for the 4 NSCLC lines examined consisting of 2 with wild-type p53, A549 and H460, and 2 that are null for p53, H1299 and Calu-6, were generated (Fig. 1A). Lines with defective p53, H1299 and Calu-6, were significantly radiosensitized but lines with wild-type p53, A549 and H460, were not and this pattern extended to 2 breast cancer lines and 2 prostate cancer lines (Supplementary Fig. S2). The degree of radiosensitization was quantified from the survival curves in 2 different ways; by comparing the surviving fractions at the radiation dose of 2 Gy (SF_2) and by calculating the dose enhancement factor (DEF), that is, the ratio of radiation doses to achieve 10% survival. The DEF values for all of the cell lines examined are provided in Table 1. SF_2 is a particularly relevant parameter in the context of clinical radiotherapy, as 2 Gy is the typical dose given on a daily basis. Some of the p53-defective cell lines had substantial and significant changes in SF_2 values. For example, for H1299 cells, SF_2 was reduced from 0.65% \pm 0.0496% in the control to 0.42% \pm 0.0777% ($P < 0.01$) by MK-1775. Such a radiosensitizing effect would be projected to result in 6 additional logs of cell kill if repeated for 30 fractions.

MK-1775 at the concentration of 200 nmol/L was slightly toxic to these cell lines, typically lowering PE by about 20%. For example, for the 2 cell lines used in subsequent experiments, the PE for A549 cells was reduced from 89.5% \pm 1.1% in controls to 72.1% \pm 1.0% in MK-1775-treated cells, and in H1299 cells, PE was reduced from 78.1% \pm 2.9% to 58.5% \pm 4.4% by MK-1775. These effects were typical for the other lines tested, independently of their p53 status, with the exception of the 2 cell lines derived from normal tissues, CCD-16 (normal lung fibroblasts) and MCF-10A (normal breast epithelial cells); PE was not affected by MK-1775 in these cell lines. Moreover, neither of these 2 lines was radiosensitized by MK-1775 (Supplementary Fig. S2 and Table 1).

Although the correlation shown in Table 1 between p53 status of a cell line and its radiosensitization by MK-1775 was evident for the panel of 8 tumor and 2 normal cell lines, we tested this relationship further using a cell line in which p53 expression is under exogenous control. Thus, we tested a cell line that we have reported on previously (24); H1299 cells that had been transfected with a Pon A-inducible p53 construct (23). Immunoblot analysis (Fig. 1C) showed that this cell line did not express p53

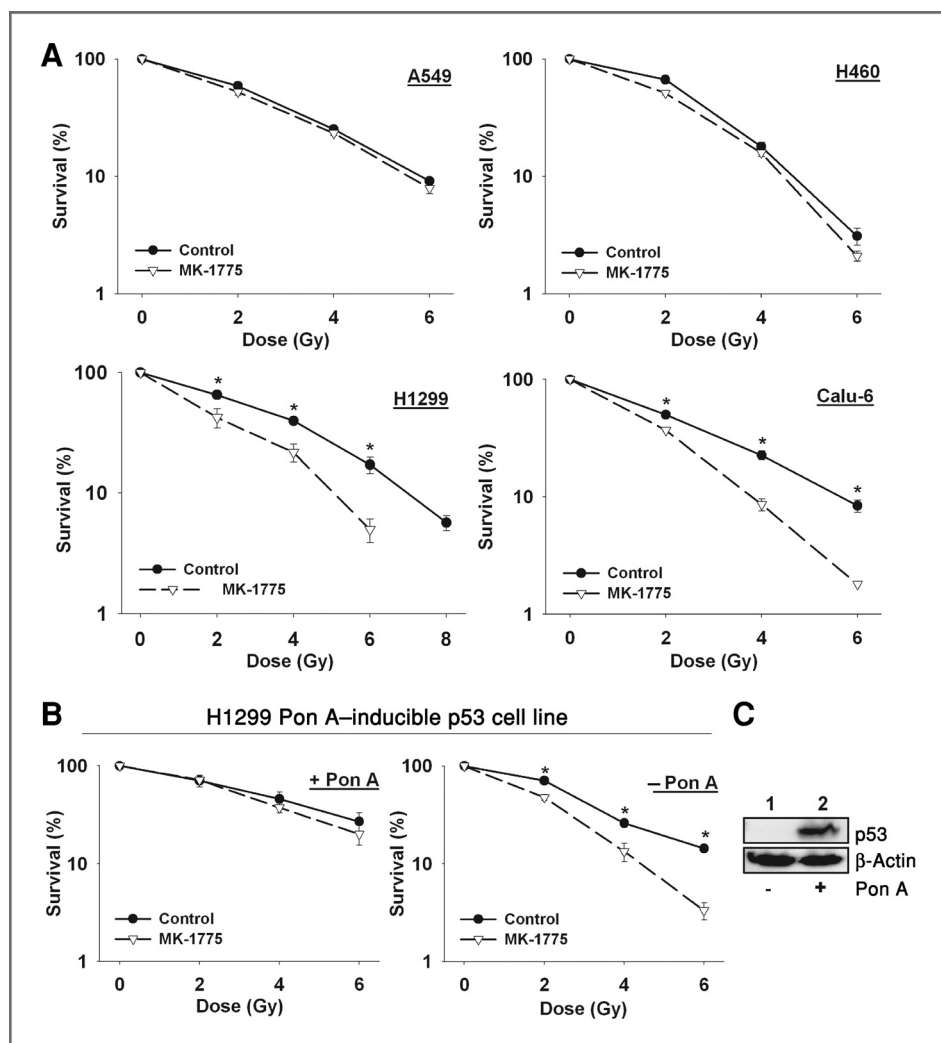


Figure 1. MK-1775 radiosensitizes NSCLC cells in a p53-dependent manner. **A**, clonogenic survival curves for A549 and H460 (both p53 wild-type) and H1299 and Calu-6 (both p53 defective) cells treated or not with 200 nmol/L of MK-1775 for 1 hour prior to irradiation followed by an additional 18 hours of postirradiation incubation in MK-1775 containing medium. The results shown represent the average of 3 or more independent determinations. Error bars are shown when larger than the symbol plotted and represent the standard error. *, $P < 0.05$. **B**, clonogenic survival curves for H1299 cells harboring a Pon A-inducible p53 expression vector treated or not with MK-1775 (as in **A**). Cells were treated with Pon A (5 μ mol/L) for 24 hours prior to irradiation (left) or not (right). The results shown represent the average of 3 or more independent determinations. Error bars are shown when larger than the symbol plotted and represent the standard error. *, $P < 0.05$. **C**, immunoblot showing p53 expression in H1299 cells harboring a Pon A-inducible p53 expression vector. Cells were treated or not with Pon A for 24 hours prior to harvest.

when cultured in medium without Pon A but robustly expressed it when cultured for 24 hours with Pon A. Clonogenic survival analysis of this cell line confirmed the p53 dependency of radiosensitization by MK-1775; radiosensitization was suppressed in these H1299 cells when p53 expression was induced by Pon A treatment compared with the radiosensitization seen when Pon A treatment was withheld (Fig. 1B).

MK-1775 abrogates the radiation-induced G₂ block in a p53-dependent manner by accelerating p53-defective cells into mitosis prematurely

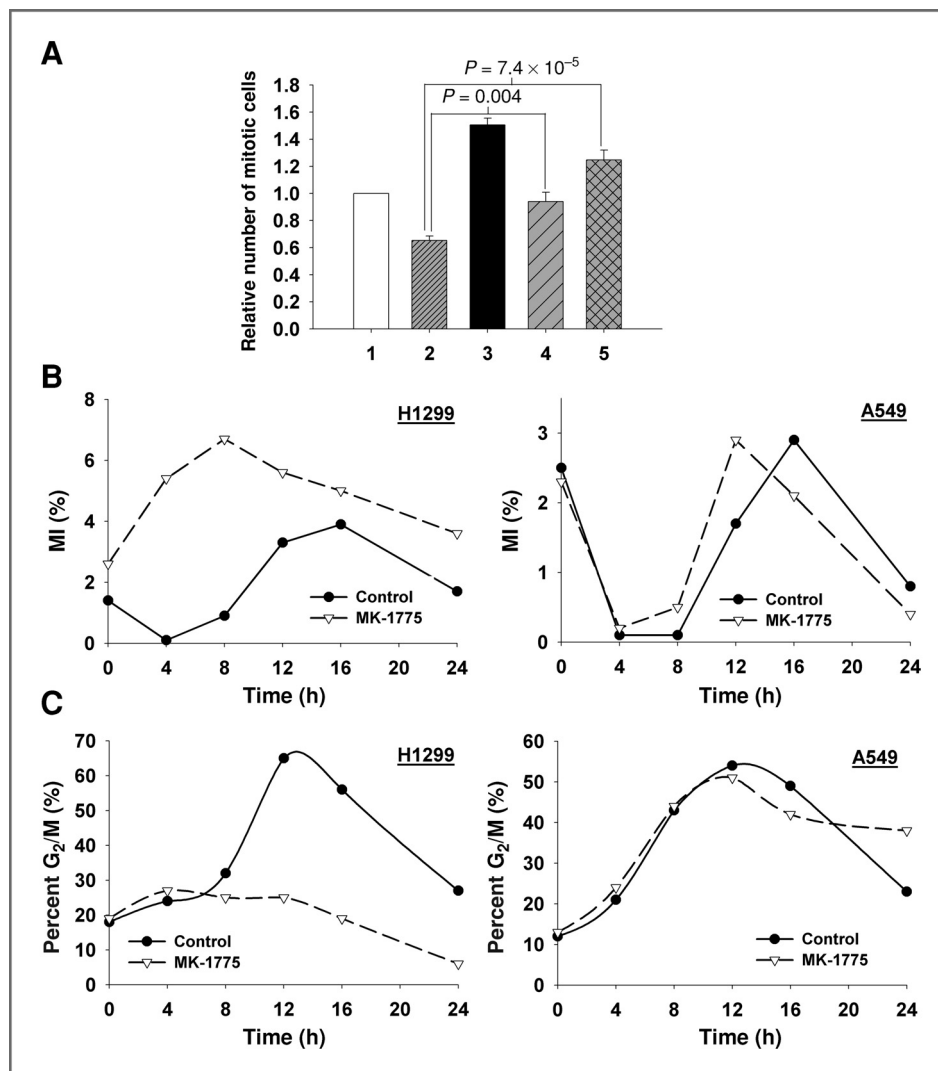
We analyzed the effect of MK-1775 on cell-cycle progression following irradiation in H1299 cells to determine whether abrogation of the G₂ block explained the radiosensitization effect of MK-1775 in this cell line. First, we conducted mitotic trap experiments. H1299 cells were treated with 200 nmol/L MK-1775 for 1 hour, irradiated with 4 Gy, and then incubated for 4 hours in medium containing nocodazole and MK-1775. These samples were compared with control samples consisting of nocodazole

alone (4 hours), MK-1775 and nocodazole (4 hours), 4 Gy and nocodazole (4 hours), and 4 Gy followed by MK-1775 and nocodazole (4 hours). At the end of the nocodazole treatment, the mitotic cells (which detach and float in the

Table 1. The DEF values calculated from the survival curves shown in Figure 1A and Supplementary Figure S2 for the p53-defective and p53 wild-type cell lines

p53 wild-type		p53 defective	
Cell line	DEF	Cell line	DEF
A549	1.0	H1299	1.4
H460	1.0	MDA-MB-231	1.3
MCF-7	1.0	PC3	1.2
LNCaP	<1	Calu-6	1.5
CCD-16	1.0		
MCF-10A	1.0		

Figure 2. MK-1775 abrogates the radiation-induced G₂ block and prematurely accelerates cells into mitosis. **A**, H1299 cells were irradiated with 4 Gy and the cells were then incubated in medium containing nocodazole for 4 hours. MK-1775 (200 nmol/L) was added either 1 hour before irradiation or immediately thereafter. The mitotic cells were selectively harvested at the end of the 4-hour nocodazole treatment by gentle shaking and counted. 1, nocodazole alone; 2, 4 Gy + nocodazole; 3, MK-1775 + nocodazole; 4, 4 Gy + MK-1775 + nocodazole; and 5, MK-1775 for 1 hour followed by 4 Gy + MK-1775 + nocodazole. The values shown represent the average of 5 independent experiments. Error bars represent the standard error. The counts for samples 2 to 5 were normalized to the nocodazole alone sample in each experiment. **B** and **C**, asynchronously growing H1299 and A549 cells were treated with MK-1775 for 1 hour or not and then irradiated with 7.5 Gy. Samples were taken as a function of time thereafter and analyzed by flow cytometry for mitosis (**B**) on the basis of p-HH3 and for percent G₂/M (**C**) on the basis of DNA content. Drug treatment was continued after irradiation in the MK-1775-treated cells.



medium) were gently collected for each sample and counted. That these cells were mitotic was validated by cytopins and Giemsa staining; the mitotic index (MI) was typically greater than 95%. The results, depicted in Figure 2A, show that MK-1775 alone accelerated unirradiated cells into mitosis compared with the nocodazole alone control. Cells irradiated with 4 Gy displayed a reduced level of mitotic cells compared with the control consistent with a radiation-induced G₂ block, but the block was reversed when MK-1775 was present during the postirradiation nocodazole treatment and reversed to an even greater extent, that is, above the nocodazole only control, when the cells were pretreated with MK-1775 for 1 hour prior to irradiation and postirradiation incubation in nocodazole plus MK-1775.

To validate that the results in Figure 2A actually represented changes in MI, a similar set of samples was generated except that all of the cells in the dishes were harvested at the end of the nocodazole treatment and the proportion of cells in mitosis was ascertained on the basis of p-HH3

stained cells as detected by flow cytometry. In this case, the response of H1299 cells was compared with A549 cells. The results (Supplementary Fig. S3) show that MK-1775 accelerates unirradiated A549 and H1299 cells into mitosis when compared with the nocodazole control and, in both A549 and H1299 cell lines, 4 Gy followed by a 4-hour incubation with nocodazole resulted in an MI lower than the level observed in the nocodazole only control indicative of the G₂ block induced by radiation in these cells. However, a 1-hour pretreatment with MK-1775 followed by 4 Gy and then a 4-hour incubation in MK-1775 + nocodazole accelerated H1299 cells into mitosis compared with the radiation alone sample, illustrating abrogation of the G₂ block as was observed in the prior experiment. A similar result was not seen in the p53 wild-type A549 cells where treatment with MK-1775 resulted in only a small increase in MI following irradiation but to a level below the MK-1775 and radiation alone controls indicating that the G₂ block in these cells is substantially maintained (Supplementary Fig. S3).

We also determined whether the radiosensitizing effects of MK-1775 correlated with abrogation of the radiation-induced G₂/M block in asynchronously growing cells. A549 and H1299 cells were treated or not with 200 nmol/L MK-1775 for 1 hour, irradiated with 7.5 Gy, returned to MK-1775-containing medium or not, and then harvested at 4-hour intervals for up to 24 hours. Cell-cycle arrest as a function of time was determined on the basis of MI by p-HH3 staining and G₂/M-associated DNA content, both assessed by flow cytometry. For both cell lines, irradiation alone caused a prompt decline in MI that reached a nadir by 4 hours (Fig. 2B). Over time, MI recovered and peaked about 16 hours after irradiation. The pattern was quite different for the 2 cell lines for the combination of MK-1775 and radiation. In H1299 cells, treatment with MK-1775 completely abrogated the decline in MI seen for irradiation alone and, instead, accelerated irradiated cells into mitosis, peaking about 8 hours after irradiation. This effect was not seen in the A549 cells; the initial decline in MI after irradiation was identical whether the cells were treated with MK-1775 or not; and cells responding to both treatments reached a nadir of 0% MI at 4 hours.

The results for the assessment of G₂/M (Fig. 2C) were consistent with those seen for the MI above. H1299 cells treated with radiation alone accumulated in G₂/M over time peaking at 12 hours after irradiation, consistent with a radiation-induced G₂ block. In the H1299 cells that were treated with MK-1775 + radiation, the cells continued to progress through G₂/M with no substantial accumulation. MK-1775 did not substantially alter the accumulation of irradiated A549 cells in G₂/M compared with that seen for radiation alone. Overall, these results are consistent with a p53-dependent abrogation of the radiation-induced G₂ block by MK-1775 in p53-defective cells. Flow cytometric profiles for some of the key time points in Figure 2C are provided in Supplementary Figure S4.

Abrogation of the G₂ block with MK-1775 causes p53-defective cells to enter mitosis and into the next cell cycle harboring radiation-induced DNA lesions

The radiosensitizing effect of MK-1775 could be explained if p53-defective cells enter mitosis prematurely and progressed into the next cell cycle before they completed repair of the radiation-induced DNA damage. Unrepaired DNA lesions, especially double-strand breaks (DSB), present at the time of mitosis would in that case be expected to have lethal consequences. To test this hypothesis, H1299 and A549 cells growing on coverslips were treated with MK-1775 for 1 hour or not, irradiated with 1 Gy, and trapped in mitosis with nocodazole for 4 hours. The dose of 1 Gy was used in this experiment due to the sensitivity of γ -H2AX foci detection. The mitotic cells in the samples were identified on the basis of their distinct morphology and γ -H2AX foci were scored in these mitotic cells by immunofluorescent staining as indicators of radiation-induced DNA damage, specifically DSBs. To underscore the relative influence of the 1-hour preirradiation treatment with MK-1775, cells treated with this protocol

were compared with cells that only received drug during the postirradiation incubation. The results, shown in Figure 3A, indicate that for both cell lines, cells that enter mitosis within 4 hours after irradiation harbor unrepaired DSBs. When MK-1775 was added to the cultures immediately after irradiation, this effect was not increased. However, mitotic H1299 cells that received a 1-hour preirradiation treatment followed by continued incubation with MK-1775 harbored significantly more DSBs compared with radiation alone ($P < 0.01$), indicating that MK-1775, due to its abrogation of the G₂ block, allows irradiated cells to prematurely enter mitosis harboring unrepaired DSBs. MK-1775 treatments did not similarly affect the levels of γ -H2AX foci in the A549 cells. For both cell lines, there was a slight increase in γ -H2AX foci in unirradiated cells that were treated with MK-1775, consistent with the toxicity of the drug on these cells and suggesting that the premature entry of cells into mitosis seen with this drug, that is, Figure 2A and Supplementary Figure S3, may lead to lethal events due to the incomplete repair of DNA replication errors. Representative photomicrographs illustrating the presence of γ -H2AX foci in H1299 cells following these different treatments are presented in Supplementary Figure S5.

In a follow-up experiment examining the induction of micronuclei, the same protocol was followed except that the cells were grown in 100-mm culture dishes irradiated with 4 Gy, and, after the 4-hour incubation in nocodazole, the mitotic cells were preferentially harvested by gentle shaking and replated onto coverslips in fresh medium without nocodazole or MK-1775. After 18 hours of incubation, the coverslips were collected, stained with DAPI, and scored for micronuclei, the presence of cells with chromosome bridges was also noted. In both H1299 and A549 cells, the incidence of micronuclei increased with radiation alone compared with unirradiated control. Treatment of H1299 cells with MK-1775 led to substantially increased numbers of micronuclei compared with radiation alone, with this effect being more robust in the cells that were treated with MK-1775 1 hour before and after irradiation compared with cells that received the drug only after irradiation (Fig. 3B). H1299 cells that were treated with MK-1775 both before and after irradiation also had substantial numbers of chromosome bridges compared to the other samples (data not shown). In A549 cells, MK-1775 given after irradiation led to increased numbers of micronuclei over the radiation alone control but to a lesser degree compared with H1299 cells. This effect was not increased by adding the drug before irradiation. Representative photomicrographs illustrating the presence of micronuclei in H1299 cells following these different treatments are presented in Figure 3D.

To confirm that MK-1775 affects its *wee1* target in both A549 and H1299 cells, we treated cells for 1 hour with 200 nmol/L and assessed the levels of p-cdc2 by Western blotting. The results (Fig. 3C) indicated that MK-1775 leads to a dephosphorylation of cdc2 downstream of *wee1* in both A549 and H1299 cells. This observed decrease in p-cdc2 below control levels is presumably due to the increased dephosphorylation of Tyr15 on cdc2 by cdc25 when the

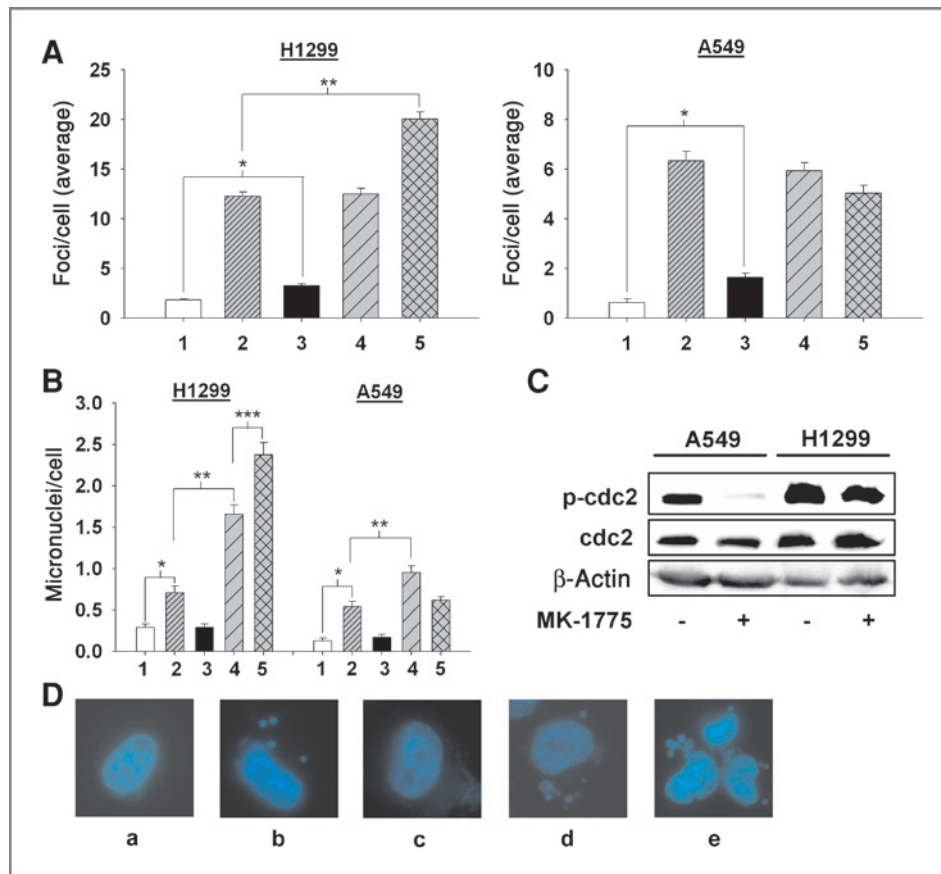


Figure 3. Cells prematurely accelerated into mitosis by MK-1775 harbor unrepaired DNA DSBs and undergo mitotic death. **A**, H1299 and A549 cells growing on coverslips were irradiated with 1 Gy and then incubated in medium containing nocodazole for 4 hours. MK-1775 (200 nmol/L) was added either 1 hour before irradiation or immediately thereafter. At the end of the 4-hour nocodazole treatment, the mitotic cells (identified by their distinctive morphology) were analyzed for DSBs on the basis of γ -H2AX foci as detected by immunofluorescence. 1, nocodazole alone; 2, 1 Gy + nocodazole; 3, MK-1775 + nocodazole; 4, 1 Gy + MK-1775 + nocodazole; and 5, MK-1775 for 1 hour followed by 1 Gy + MK-1775 + nocodazole. Error bars represent the standard error. *, **, $P < 0.05$. **B**, cells growing in 100-mm dishes were treated (as in **A**) except that the dose of radiation was 4 Gy. At the end of the 4-hour nocodazole treatment, mitotic cells were harvested by gentle shaking and replated on coverslips in medium without nocodazole for 18 hours. Cells were then harvested, stained with DAPI, and analyzed for the presence of micronuclei. Error bars represent the standard error. *, **, ***, $P < 0.05$. **C**, Western blot for A549 and H1299 cells treated for 1 hour with 200 nmol/L of MK-1775 and analyzed for p-cdc2 expression. **D**, representative photomicrographs of H1299 cells treated (as in **B**) illustrating the presence of micronuclei. **a**, nocodazole alone; **b**, 4 Gy + nocodazole; **c**, MK-1775 + nocodazole; **d**, 4 Gy + MK-1775 + nocodazole; and **e**, MK-1775 (1 hour) + 4 Gy + MK-1775 + nocodazole.

balance of wee1 and cdc25 competing activities is upset by inhibition of wee1 by MK-1775. This effect was recapitulated in H460 and Calu-6 cells (Supplementary Fig. S6A). In addition, we tested whether p-cdc2 levels were suppressed in cells irradiated with 7.5 Gy and incubated with MK-1775 after irradiation. Although it was difficult to observe a substantial activation of wee1 activity by radiation due to the fact that almost all of the cdc2 is normally already phosphorylated in asynchronously growing cells (cdc2 is normally dephosphorylated by cdc25 during the cell cycle only in the small subpopulation of G_2 phase cells about to enter mitosis), the Western blot analyses shown in Supplementary Figure S6B and C indicate that MK-1775 leads to a dephosphorylation of p-cdc2 in irradiated H1299, A549, H460, and Calu-6 cells independently of their p53 status.

We also tested whether radiosensitization by MK-1775 involved enhanced apoptosis. H1299 cells were treated for

1 hour with 200 nmol/L MK-1775, irradiated with 7.5 Gy, incubated for an additional 18 hours in MK-1775, and harvested for assessment of apoptosis at 24, 48, and 72 hours postirradiation. Apoptosis levels were determined on the basis of Annexin V staining and sub- G_1 DNA content, both assessed by flow cytometry. The results indicated that the dose of 7.5 Gy induced levels of apoptosis of only about 5% above control at any time point, and these levels of apoptosis were not significantly enhanced by MK-1775 (data not shown).

MK-1775 enhances H1299 xenograft tumor response to fractionated radiotherapy

On the basis of the substantial radiosensitization by MK-1775 in the p53-defective NSCLC cell lines (Table 1), we determined whether this effect extended to the *in vivo* situation. We conducted a series of experiments to examine

this question using xenograft tumors growing in nude mice made from one of the p53-defective NSCLC lines and treated with the combination of MK-1775 and external beam radiation where tumor growth delay was used as the endpoint for analysis. The Calu-6 cell line was chosen for this study on the basis of its substantial radiosensitization by MK-1775 in the *in vitro* survival curve analysis (Fig. 1A and Table 1). Various treatment protocols were investigated including testing different sequences of drug and radiation, different doses of drug, and different radiation fractionation schemes. Many of these protocols indicated that tumor growth delay was significantly enhanced by the drug/radiation combination compared with radiation alone. The greatest response was observed when tumors were irradiated twice a day with 1 Gy for 5 days and 60 mg/kg given twice a day on the same days as irradiation. The results of this experiment are presented in Figure 4. The EF for this treatment protocol was 3.2 ($P < 0.01$). These results underscore the importance of sequencing the drug and radiation treatment close in time and show that the radiosensitizing effect of MK-1775 extends to the *in vivo* setting.

Discussion

In this study, we investigated the radiosensitizing abilities of a novel, potent, and highly selective inhibitor of the *wee1* kinase, MK-1775. Although previous reports have shown that MK-1775 sensitizes p53-defective tumor cells to other DNA-damaging agents such as gemcitabine and cisplatin (19), its radiosensitizing properties have not been previously shown. We focused our tests of MK-1775 on cell

lines derived from 3 types of human tumors, that is, NSCLC, breast, and prostate, where radiotherapy typically plays a key role in the management of patients with these tumors and where improvements in radioresponse in these disease sites would be expected to provide clinical benefit. As shown in Figure 1A and Supplementary Figure S2 and summarized in Table 1, 4 p53-defective human tumor cell lines were radiosensitized by nanomolar concentrations of MK-1775, whereas 4 tumor cell lines with wild-type p53 and 2 cell lines of normal tissue origin were not. This comparison of p53-defective and p53 wild-type cell lines makes a convincing argument that the radiosensitizing effect of MK-1775 is p53 dependent. However, to bolster that argument, we also tested H1299 cells in which p53 expression had been restored using a Pon A-inducible vector. These results (Fig. 1B) confirmed the p53 dependence of radiosensitizing effect of MK-1775.

Only 1 other small molecule *wee1* kinase inhibitor has been previously reported. In 2001, Wang and colleagues described the development of the *wee1* inhibitor PD166285 and showed that it abrogated the G₂ checkpoint and radiosensitized p53-defective human colon carcinoma cells *in vitro* (18). In a more recent study, PD166285 was shown to radiosensitize *wee1*-overexpressing glioblastoma cells that were not p53 defective by abrogating their radiation-induced G₂ checkpoint upon which they had become dependent (25). Thus, the radiosensitizing effects of PD166285 and the results with MK-1775 reported here are consistent and once again illustrate the profound importance of the G₂ checkpoint in mediating the response of cells to radiation (11). Although, this has been well understood for many years, the identification of *wee1* as a viable drug target offers a unique opportunity for enhancing the therapeutic effects of DNA-damaging agents such as radiation in p53-defective and *wee1*-overexpressing tumor cells (26, 27).

Following our appreciation of the fact that the 1-hour preirradiation treatment added significant additional radiosensitization to the 18-hour postirradiation protocol, we conducted additional experiments to understand its impact. Apparently, this effect is due to a requirement for a finite amount of time for MK-1775 to affect its target and we showed that MK-1775 leads to the dephosphorylation of *cdc2*, substrate of *wee1*, by *cdc25* within 1 hour (Fig. 3C). Then, due to this dephosphorylation of *cdc2*, the drug accelerates both unirradiated and irradiated cells into mitosis prematurely as shown in the mitotic trap experiments (Fig. 2A and Supplementary Fig. S3). In asynchronously growing cells, irradiation promptly blocked cells in G₂ causing a sharp decline in mitotic cells (Fig. 2B). However, this block was abrogated by MK-1775 in H1299 cells leading to a significantly higher level of γ -H2AX foci in cells trapped in mitosis and a higher level of micronuclei in cells allowed to progress into the next cell cycle when treatment with MK-1775 commenced 1 hour prior to irradiation compared with initiating drug treatment immediately after irradiation (Fig. 3A and B). It is these increased levels of DSBs and their subsequent conversion to micronuclei in

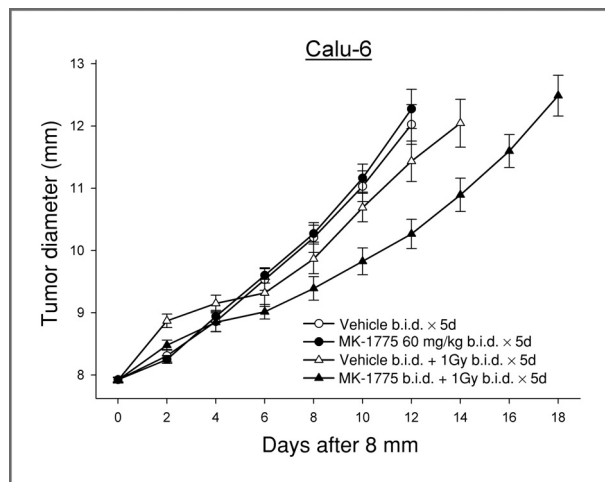


Figure 4. MK-1775 radiosensitized Calu-6 xenograft tumors growing in nude mice. Xenograft tumors made using p53-null Calu-6 cells were treated with MK-1775, 60 mg/kg twice daily (b.i.d.), or radiation, 1 Gy twice daily, or the combination for 5 consecutive days starting when the tumors reached 8 mm in diameter. Tumor growth was followed until the tumors reached 12 mm in diameter and the time for the tumors to grow from 8 to 12 mm was calculated for the treated groups and compared with the growth in the untreated controls. The error bars are the standard error of the mean tumor diameter for the group on that day and there were 8 mice in each group.

the next cell cycle observed when irradiated cells are prematurely accelerated into mitosis that explain radiosensitizing effect of MK-1775. Thus, radiosensitization of MK-1775 is not due to an inhibition of DNA repair but an abrogation of the extra repair time that would normally be allowed during a G₂ block. When the drug treatment is only given after irradiation, a proportion of cells may already be blocked in G₂ and cannot be radiosensitized by MK-1775. It is also conceivable that MK-1775, when given 1 hour prior to irradiation accelerates a proportion of unirradiated cells into a more radiosensitive phase of the cell cycle (i.e., mitosis). Although PD166285 was shown to accelerate irradiated cells into mitosis prematurely, the premature acceleration of unirradiated cells into mitosis by a wee1 inhibitor, as shown here for MK-1775, has not been reported previously to our knowledge. This may be an important feature of this drug as it may partially explain its activity as a single agent.

The question remains that why is MK-1775 ineffective as a radiosensitizer in tumor cells and normal cells that have wild-type p53 status? The answer appears to involve a critical role for p53 in governing the G₂/M transition in DNA-damaged cells in addition to its well-known role in blocking such cells in G₁ phase. What is known of this role for p53 in the G₂/M transition has recently been reviewed and may involve a number of possible mechanisms (28). Following DNA damage, activation of p53 leads to induced expression of p21/waf1, GADD45, and 14-3-3 σ . It has been proposed that each of these proteins may bind to the cdc2/cyclin B complex and modulate its function in such a manner as to inactivate it thereby causing an arrest of cells in G₂ independently of the action of the wee1 kinase (29). However, in this case, a finite amount of time would be required for this p53-induced expression and, therefore, a small proportion of irradiated cells may escape a G₂ block and progress into mitosis. Such an effect may explain the small increase in mitotic cells (Supplementary Fig. S3) and micronuclei (Fig. 3B) observed in p53 wild-type A549 cells following MK-1775 treatment.

In addition to its ability to sensitize human tumor cells to DNA-damaging agents *in vitro*, MK-1775 has similar activity against human xenograft tumors growing *in vivo*. Hirai and colleagues reported that MK-1775 enhanced antitumor efficacy of gemcitabine, carboplatin, cisplatin, and 5-FU in a model consisting of nude rats bearing WiDr human colon carcinoma xenografts (19, 21). Here, we show that this antitumor efficacy extends to NSCLC xenografts growing in nude mice treated with the combination of MK-1775 and

external beam radiation. The wee1 inhibitor, PD166285, has also been examined in combination with radiation for the treatment of glioblastoma in an orthotopic mouse model (25). The combination significantly extended the survival of the mice compared with mice treated with either agent when used alone or to untreated controls. Because of a combination of factors related to the compound (27), PD166285 has not been investigated clinically. However, MK-1775 is currently being evaluated in phase I clinical trials in combination with gemcitabine, carboplatin, or cisplatin in patients with advanced solid tumors. A preliminary report indicated that MK-1775 is well tolerated in monotherapy and in combination with chemotherapy (22).

In conclusion, we have shown that the wee1 kinase inhibitor, MK-1775, at nanomolar concentrations, potentially radiosensitizes human tumor cells derived from lung, breast, and prostate cancers in a p53-dependent manner. Lung cancer cells growing as xenograft tumors were also radiosensitized by this combination. The mechanism to explain this sensitization appears to involve a drug-induced, premature acceleration of G₂ phase cells into mitosis. Such cells harbor unrepaired DNA lesions that lead to abnormal cell divisions and cell death. These findings support the continued clinical assessment of MK-1775 in combination with DNA-damaging agents including radiation.

Disclosure of Potential Conflicts of Interest

C.A. Buser is an employee of Merck Sharp & Dohme. H. Hirai is an employee of Taiho Pharmaceutical Co. Ltd. K.A. Bridges, C. Brooks, T.A. Buchholz, J.M. Molkenkine, K.A. Mason, and R.E. Meyn received research support for this project from Merck Sharp & Dohme.

Acknowledgments

The authors acknowledge the technical assistance of David Molkenkine and Mayra Vasquez and Dr. Jack Roth for the kind gift of the H1299 cells with the Pon A-inducible p53 construct. STR DNA fingerprinting was done by the Institution's Characterized Cell Line Core.

Grant Support

The Institution's Characterized Cell Line Core was funded by cancer center support grant (NCI CA16672). The study was supported by Merck Sharp & Dohme Corp.

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Received March 14, 2011; revised July 14, 2011; accepted July 18, 2011; published OnlineFirst July 28, 2011.

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MK-1775, a Novel Wee1 Kinase Inhibitor, Radiosensitizes p53-Defective Human Tumor Cells

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Clin Cancer Res 2011;17:5638-5648. Published OnlineFirst July 28, 2011.

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