RADIATION RESEARCH **184**, 660–669 (2015)
0033-7587/15 $15.00

DOI: 10.1667/RR14171.1

# Targeting of Carbon Ion-Induced $G_2$ Checkpoint Activation in Lung Cancer Cells Using Wee-1 Inhibitor MK-1775

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**Ma, H., Takahashi, A., Sejimo, Y., Adachi, A., Kubo, N., Isono, M., Yoshida, Y., Kanai, T., Ohno, T. and Nakano, T. Targeting of Carbon Ion-induced $G_2$ Checkpoint Activation in Lung Cancer Cells Using Wee-1 Inhibitor MK-1775. *Radiat. Res.* 184, 660–669 (2015).**

**The potent inhibitor of the cell cycle checkpoint regulatory factor Wee-1, MK-1775, has been reported to enhance non-small cell lung cancer (NSCLC) cell sensitivity to photon radiation by abrogating radiation-induced $G_2$ arrest. However, little is known about the effects of this sensitizer after exposure to carbon (C)-ion radiation. The purpose of this study was therefore to investigate the effects of C ions in combination with MK-1775 on the killing of NSCLC cells. Human NSCLC H1299 cells were exposed to X rays or C ions (290 MeV/n, 50 keV/μm at the center of a 6 cm spread-out Bragg peak) in the presence of MK-1775. The cell cycle was analyzed using flow cytometry and Western blotting. Radiosensitivity was determined using clonogenic survival assays. The mechanisms underlying MK-1775 radiosensitization were studied by observing H2AX phosphorylation and mitotic catastrophe. $G_2$ checkpoint arrest was enhanced 2.3-fold by C-ion exposure compared with X-ray exposure. Radiation-induced $G_2$ checkpoint arrest was abrogated by MK-1775. Exposure to radiation resulted in a significant reduction in the mitotic ratio and increased phosphorylation of cyclin-dependent kinase 1 (Cdk1), the primary downstream mediator of Wee-1-induced $G_2$ arrest. The Wee-1 inhibitor, MK-1775 restored the mitotic ratio and suppressed Cdk1 phosphorylation. In addition, MK-1775 increased H1299 cell sensitivity to C ions and X rays independent of *TP53* status. MK-1775 also significantly increased H2AX phosphorylation and mitotic catastrophe in irradiated cells. These results suggest that the $G_2$ checkpoint inhibitor MK-1775 can enhance the sensitivity of human NSCLC cells to C ions as well as X rays.** © 2015 by Radiation Research Society

*Editor's note*. The online version of this article (DOI: 10.1667/RR14171.1) contains supplementary information that is available to all authorized users.

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## INTRODUCTION

Heavy charged particle beams are becoming an increasingly important option for cancer therapy because of their high relative biological effectiveness (RBE; mainly attributed to the end of their trajectory known as the Bragg peak) as well as their excellent dose distribution (*1–3*). Patients with early-stage non-small cell lung cancer (NSCLC) treated at the National Institute of Radiological Sciences with carbon (C)-ion radiotherapy without severe toxicities, had a 5-year overall survival and local control rates of 45–50% and 90–95%, respectively (*4–6*). However, subgroup analysis showed that the local control rate was correlated with tumor diameter (*5, 7*), and in some cases the organ at risk was too close to allow an increased radiation dose to the tumor, especially in patients with locally advanced NSCLC. These findings suggest the need for further tumor-specific enhancement of radiosensitivity to C ions using molecular targeted agents.

Exposure to ionizing radiation causes cell cycle checkpoint activation (*8*) that specifically blocks or slows the progression of cells into the next phase, thus allowing them more time to repair DNA damage. Two major cell cycle checkpoints that occur are known as the $G_1$ and $G_2$ checkpoints. The $G_1$ checkpoint is activated in a *TP53*-dependent manner. However, *TP53* mutations have been identified in approximately 50% of NSCLCs and over 70% of small cell lung cancers (*9*), making the $G_2$ checkpoint the last barrier in *TP53*-defective NSCLC cells. Furthermore, mutations in the *TP53* gene and *TP53* defects lead to radioresistance because of the critical role of the p53 protein in the radiation-induced cell death pathway. Consequently, regulatory factors involved in $G_2$ arrest have been investigated as molecular targets to enhance cancer cell radiosensitivity.

Wee-1 kinase is a regulatory factor involved in the $G_2$ checkpoint (*10*). It leads to $G_2$ checkpoint activation, thereby preventing damaged cells from entering into premature mitosis with incompletely repaired DNA damage (*11–15*). Wee-1 is overexpressed in various cancer types (*16–19*). The Wee-1 inhibitor (MK-1775) has thus been included in phase I and phase II clinical trials as a potential candidate for

molecular targeted therapy, either as monotherapy or in combination with chemotherapeutic agents (*16*). One study demonstrated that MK-1775 abrogated radiation-induced $G_2$ arrest and enhanced *TP53*-defective NSCLC cell radiosensitivity to photons by allowing cancer cells to enter mitosis prematurely, while harboring DNA lesions (*10*).

We previously demonstrated that compared to X rays, C ions induced a stronger $G_2$/M checkpoint (*20*). However, little is known about the effects of radiosensitizing agents on the effects of C ions. We therefore investigated the effects of C ions combined with MK-1775 on killing of NSCLC cells, to evaluate its radiosensitizing effect in C ion-based cancer therapies.

## MATERIALS AND METHODS

### *Cell Lines*

We used the human NSCLC cell lines H1299 (*TP53*-null), H1299/*neo* (*TP53*-null), H1299/m*p53* (mutated *TP53*) and H1299/wt*p53* (wild-type *TP53*), provided by Dr. Moshe Oren (Weizmann Institute of Science, Rehovot, Israel) and Dr. Hideki Matsumoto (University of Fukui, Fukui, Japan) (*21*). The human NSCLC cell line A549 (wild-type *TP53*) and human glioblastoma cell line A172 (wild-type *TP53*) were purchased from the American Type Culture Collection (ATCC®, Gaithersburg, MD). All cells were cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) with high glucose and L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and HEPES (10 m*M*) at 37°C in a humidified atmosphere of 5% $CO_2$.

### *Irradiation*

X-ray radiation (1.3 Gy/min) was administered using a 200 kVp X-ray generator (TITAN-225S, Shimadzu, Kyoto, Japan), at 14.6 mA with a total filtration of 0.5 mm aluminum plus 0.5 mm copper. C-ion irradiation (approximately 3 Gy/min) was performed using a 290 MeV/n synchrotron (Gunma University Heavy Ion Medical Center, Gunma, Japan), with a dose-averaged linear energy transfer of 50 keV/μm at the center of a 6 cm spread-out Bragg peak (*22*).

### *Treatments*

Cells were treated with the cell cycle $G_2$ checkpoint Wee-1 inhibitor MK-1775 (Selleck Chemicals, Boston, MA) or vehicle (dimethyl sulfoxide) control for a total of 24 h, beginning 6 h before irradiation. All inhibitors were removed and the cells were re-fed with fresh media 18 h after irradiation (*23*) (Fig. 1).

### *Cell Cycle Phase Analysis*

Cells were fixed in 70% ethanol. Propidium iodide (PI) staining involved permeabilization with 400 μl phosphate-buffered saline containing 0.1 mg/ml PI, 0.1 % Triton™ X-100 and 400 μl (0.8 mg) DNase-free RNaseA at room temperature. To determine the percentage of cells in mitosis, cells were stained with a mouse monoclonal antibody against human phospho-histone H3 (Ser10) (1:5,000; Millipore, Billerica, MA) and an Alexa Fluor® 488-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (1:400; Molecular Probes®, Eugene, OR). Samples were examined using a FACSCalibur™ (BD Biosciences, Franklin Lakes, NJ) and the data were analyzed with FlowJo software (Digital Biology, Tokyo, Japan). The slopes of the correlations between radiation dose and percentage of $G_2$/M-phase cells were calculated using KaleidaGraph 3.5 software (Synergy Software, Reading, PA). Mitotic ratio (MR) values were calculated according to the following formula: MR = percentage of phospho-histone H3-positive cells/percentage of total cells in gate.

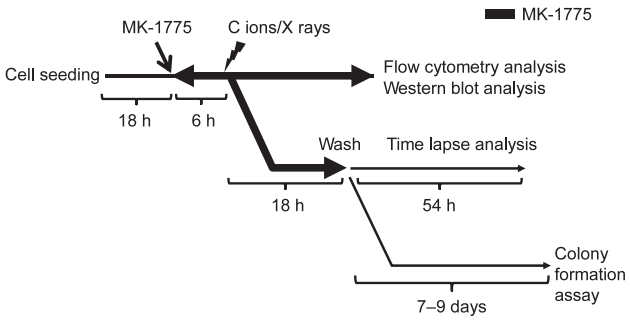


**FIG. 1.** Schematic work flow for experiments.

### *Western Blotting*

Cells were lysed in RIPA lysis buffer at different times. Western blotting was performed using the following primary antibodies: mouse monoclonal antibodies against human cyclin-dependent kinase 1 (Cdk1, 1:1,000; BD Biosciences); human tyrosine 15-phosphorylated Cdk1 (Cdk1pY15, 1:1,000; BD Biosciences); rabbit polyclonal antibody against human Wee-1 (1:1,000; Cell Signaling Technologies, Tokyo, Japan); mouse monoclonal antibody against human $p21^{WAF1}$ (1:100; Calbiochem, Darmstadt, Germany); and goat monoclonal antibody against human actin (1:1,000; Santa Cruz Biotechnology® Inc., Santa Cruz, CA). Secondary antibodies were: a horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody (1:10,000; GE Healthcare, Buckinghamshire, UK) for Cdk1 and Cdk1pY15; HRP-conjugated donkey anti-rabbit IgG antibody (1:10,000; GE Healthcare) for Wee-1; HRP-conjugated sheep anti-mouse IgG antibody (1:1,000) for $p21^{WAF1}$; and HRP-conjugated rabbit anti-goat IgG antibody (1:10,000; Zymed Laboratories Inc., San Francisco, CA) for actin. The bound antibody was visualized using an ECL™ Plus Western blotting chemiluminescence detection reagent system (GE Healthcare). The results were normalized against the intensity of actin in each sample.

### *Radiosensitivity Assays*

Cell radiosensitivity was defined using a standard colony formation assay. The percentages of surviving cells after combined irradiation and MK-1775 were normalized to the surviving fractions after MK-1775 treatment alone. Two or three flasks or petri dishes were used for each experimental point. Colonies obtained after 9 days were fixed with methanol and stained with 2% Giemsa solution. Microscopic colonies composed of more than approximately 50 cells were considered to have grown from a single surviving cell. $D_{10}$ values were determined from the surviving-fraction curve as the dose (Gy) required to reduce the surviving fraction to 10%, and were calculated using the linear-quadratic formula. The RBE values of C ions and sensitization ratio (SR) values were calculated from $D_{10}$ values according to the following formulae:

$$\mathrm{RBE} = D_{10}\ \text{of X-irradiated cells}/D_{10}\ \text{of C ion-irradiated cells};$$

$$\mathrm{SR} = D_{10}\ \text{of irradiation alone}\ /D_{10}\ \text{of combination of inhibitor and irradiation.}$$

### *DNA Damage Analysis*

Cells were fixed in 70% ethanol and stained with a mouse monoclonal antibody against human γ-H2AX (1:300; Millipore) and

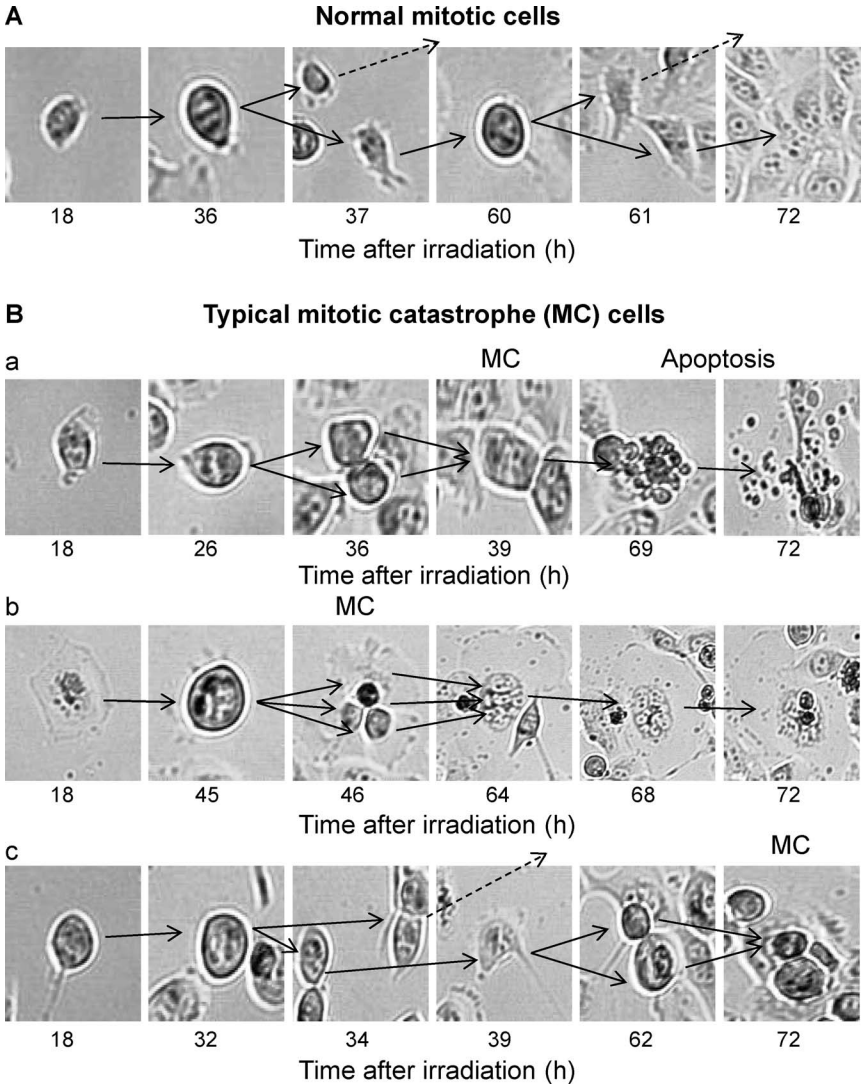


**FIG. 2.** Typical time-lapse images within 72 h after irradiation. Panel A: Normal mitotic cells. Panel B: Mitotic catastrophe (MC) cells. In mitotic catastrophe, multinucleated cells developed as a result of incomplete mitosis. Compared with normal mitosis (panel A), mitotic catastrophe (MC) cells were unable to undergo complete division, resulting in multinucleated cells (panel B). At 36 h after irradiation, this cell started to divide but the two daughter nuclei were unable to separate, and returned to the mother cell 39 h after irradiation, followed by mitotic catastrophe-related apoptosis (panel a). At 46 h after irradiation, this cell tried unsuccessfully to divide into three daughter cells, but the daughter nuclei returned to the mother cell 64 h after irradiation (panel b). Multinucleated cells appeared after incomplete mitosis that followed one normal mitosis. This cell divided into two daughter cells 34 h after irradiation, but one of these daughter cells was unable to divide normally 62 h after irradiation, creating a multinucleated cell 72 h after irradiation (panel c).

an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody. The cells were then stained with PI. Flow cytometry analysis was performed as described above.

*Mitotic Catastrophe Evaluation*

Cells were maintained in 35 mm dishes in an incubator at 37°C and 5% $CO_2$ for time-lapse analysis. Time-lapse images and videos were captured at 5 min intervals for 54 h using a JuLI FL Fluorescence live cell movie analyzer (AR Brown, Tokyo, Japan) starting from when the media was changed at 18 h after irradiation (Fig. 1). Cells in the first image (18 h after irradiation) were counted as the total cell number for each sample, and individual cells followed until 48 h after irradiation. A minimum of 100 cells were traced and scored for each experimental condition. Mitotic catastrophe was defined as the percentage of cells undergoing abnormal mitosis based on morphological evidence of multinucleated cells. Morphological identification of mitotic catastrophe is explained in detail with accompanying images shown in Fig. 2.

*Statistical Analysis*

All values were presented as the mean ± standard deviation (SD), with $n$ indicating the number of independent experiments. Data were analyzed statistically using Student's $t$ tests. A $P$ value of $<0.05$ was considered significant.

**TABLE 1**
**$D_{10}$ Values and Sensitization Ratios for Different Concentrations of MK-1775 in H1299 Cells**

| | $D_{10}$ | | Sensitization ratio | |
|---|---|---|---|---|
| MK-1775 (n*M*) | Carbon ions | X rays | Carbon ions | X rays |
| 0 | 3.74 ± 0.75 | 7.68 ± 0.30 | 1 | 1 |
| 100 | 3.34 ± 0.43 | 6.20 ± 0.57 | 1.14 ± 0.15 | 1.25 ± 0.12 |
| 250 | 3.11 ± 0.17 | 6.30 ± 0.18 | 1.21 ± 0.07 | 1.22 ± 0.04 |
| 500 | 3.11 ± 0.36 | 5.92 ± 0.37 | 1.21 ± 0.15 | 1.30 ± 0.08 |

*Note*. $D_{10}$ = dose resulting in 10% cell survival.

## RESULTS

### *MK-1775 Abrogates C-Ion-Induced $G_2$ Arrest and Phosphorylation of Cdk1*

We determined the potential effect of MK-1775 on the abrogation of C-ion-induced $G_2$ cell cycle checkpoint arrest using flow cytometry analysis. Based on the results of the radiation dose-dependent $G_2$/M percentages shown in Fig. 3A, we used 5 Gy C ions, and 5 and 10 Gy X rays, which resulted in increased numbers of $G_2$/M-phase cells over a similar time course up to 12 h, followed by normalization by 24 h, independent of radiation dose. $G_1$- and S-phase cells decreased 12 h after irradiation and recovered 24 h after irradiation (Fig. 3B). However, C-ion irradiation resulted in an approximately 2.3-fold increase in the number of $G_2$/M-phase cells compared to X rays, based on the slope ratio, (slopes for C ions and X rays: 10.08 ± 0.51 and 4.44 ± 0.33, respectively; $P = 0.0003$). In contrast, $G_1$- and S-phase cells decreased in a dose-dependent manner for both types of radiation (Fig. 3A). In addition, radiation-induced $G_2$ arrest was almost completely abrogated by approximately 100 n*M* MK-1775, and the effect was maximal and concentration independent at concentrations ≥250 n*M*. The radiation-induced decreases in $G_1$- and S-phase cells were recovered by MK-1775 (Fig. 3C).

Although we confirmed increases in $G_2$/M-phase cells after irradiation, it was not possible to distinguish between $G_2$- and M-phase cells by PI staining. We therefore analyzed M-phase-specific phosphorylation of histone H3 to confirm the $G_2$ checkpoint. Exposure to 5 Gy C ions and 10 Gy X rays resulted in a significant reduction in the mitotic ratio at 3 h after irradiation, reflecting the onset of $G_2$ arrest. Pretreatment with MK-1775 pushed $G_2$-phase cells into M phase after irradiation by abrogation of radiation-induced $G_2$ arrest, as shown by an increased mitotic ratio (Fig. 4A). In addition, it has been shown that phosphorylation and inactivation of Cdk1 is important in activating the regulator of Wee-1-induced $G_2$ arrest (*15*). We therefore used Western blotting to determine the potential inhibitory effect of MK-1775 on Cdk1 phosphorylation. Increased phosphorylation of Cdk1 was observed at 12 h after irradiation with 5 Gy C ions or 10 Gy X rays. Strikingly, pretreatment with MK-1775 (100 n*M*) 6 h before irradiation attenuated Cdk1 phosphorylation, further supporting the role of MK-1775 in $G_2$ checkpoint abrogation (Fig. 4B).

### *MK-1775 Enhances C-Ion-Induced Cell Killing in NSCLC Cells*

Clonogenic survival assays were conducted after 24 h exposure to graded concentrations of MK-1775. Exposure of *TP53*-null cells to <100 n*M* MK-1775 resulted in minimal cytotoxicity, with a surviving fraction of nearly 90%, while 150 and 200 n*M* MK-1775 reduced survival to approximately 70% and 50%, respectively (Fig. 5A). To clarify if MK-1775 could enhance radiosensitivity to C ions, we examined the surviving fraction using a clonogenic survival assay and 100 n*M* MK-1775, because, as indicated above, this concentration produced very little independent cytotoxicity (surviving fraction >90%). Exposure of *TP53*-null H1299 cells to 100 n*M* MK-1775 resulted in similar increases in radiosensitivity when combined with either 5 Gy C ions (Fig. 5B) or 10 Gy X rays (Fig. 5C). This was supported by the calculated sensitization ratio (SR) values (1.14 and 1.25, respectively; Table 1). Based on the surviving fraction curve, the RBE of the C ions was 2.4 at $D_{10}$, indicating greater ability to induce DNA damage relative to X rays. This RBE corresponds with the increase in $G_2$ arrest (2.3-fold) induced by C ions. Sensitization to C ions and X rays at $D_{10}$ almost reached saturation at concentrations of 250 n*M* and 100 n*M* MK-1775, respectively (Table 1). Additionally, there was no significant difference in SR between C ions and X rays at the same biological dose (Table 1).

We also investigated the effect of MK-1775 in H1299/*neo*, H1299/m*p53* and H1299/wt*p53* cell lines to clarify if the sensitizing effect of MK-1775 to C ions occurred in a *TP53*-dependent manner. Upregulation of $p21^{WAF1}$ encoded by the *WAF1/Cip1* gene is directly involved in p53 signaling (*24*). We therefore assessed *TP53* function in cell lines by analyzing $p21^{WAF1}$ expression level. Reduced $p21^{WAF1}$ expression levels in *TP53*-null H1299, H1299/*neo* and H1299/m*p53* cell lines were confirmed by Western blotting, whereas $p21^{WAF1}$ expression was increased 6 h after 5 Gy X-ray irradiation in H1299/wt*p53* cells and positive control human glioblastoma A172 cells (Fig. 5D). MK-1775 thus sensitized not only H1299/*neo* and H1299/m*p53* cells, but also H1299/wt*p53* cells to C ions, independent of *TP53* status (Fig. 5E). In addition, MK-1775 sensitized *TP53*-proficient human lung cancer A549 cells (Supplementary Fig. S1; http://dx.doi.org/10.1667/RR14171.1.S1).

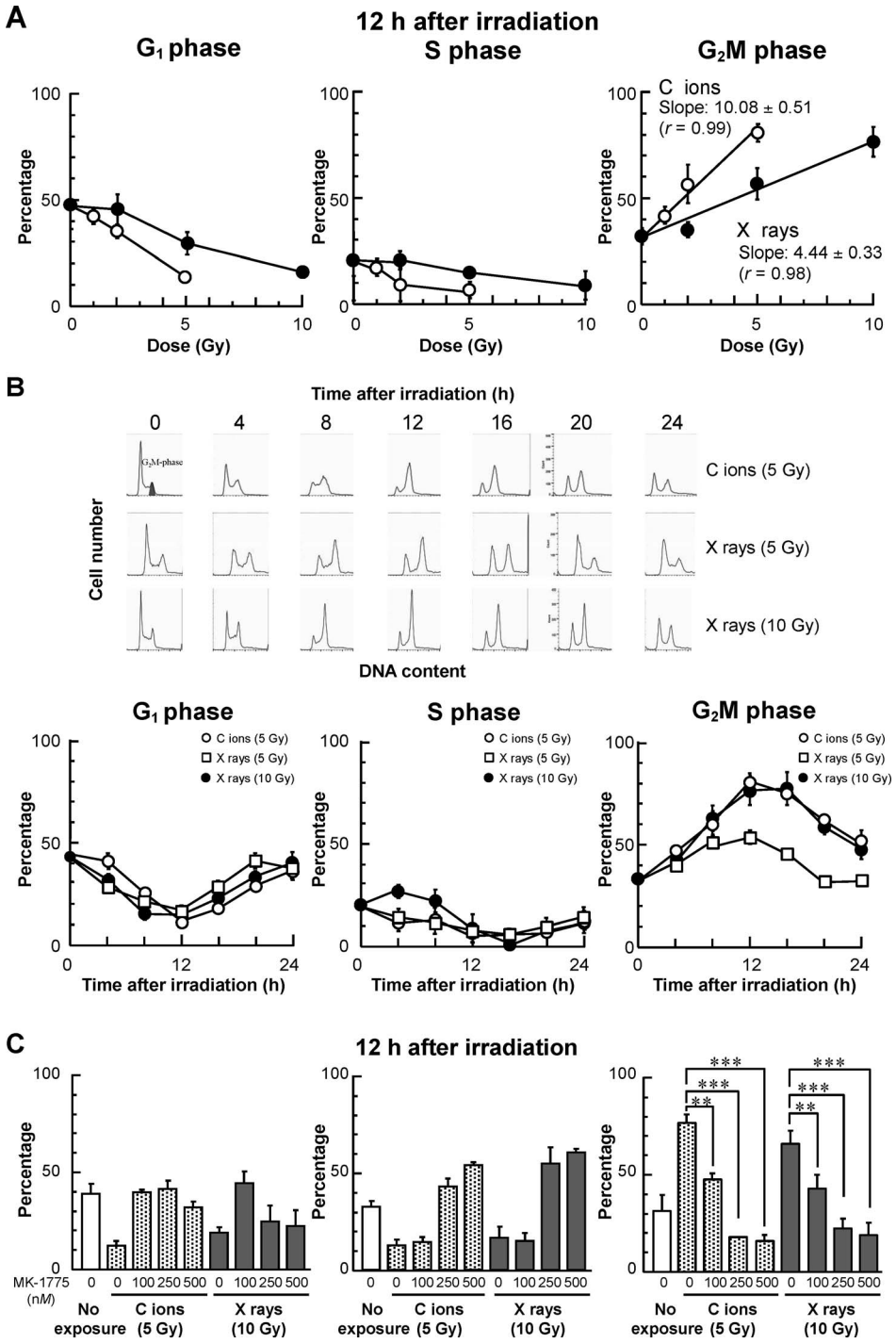


**FIG. 3.** Effect of MK-1775 and irradiation on cell cycle regulation in *TP53*-null H1299 cells. Panel A: Radiation dose-dependent changes in percentages of $G_1$-, S- and $G_2$/M-phase cells 12 h after irradiation. Panel B: Time-dependent changes in percentages of $G_1$-, S- and $G_2$/M-phase cells after irradiation. Panel C: MK-1775 concentration-dependent changes in percentages of $G_1$-, S- and $G_2$/M-phase cells at 12 h after irradiation. Results are shown as mean ± SD of three independent experiments. Data were analyzed using Student's *t* test (**$P < 0.01$; ***$P < 0.001$).

*MK-1775 Increases C-Ion-Induced H2AX Phosphorylation and Mitotic Catastrophe*

The potential of MK-1775 to influence DNA repair ability was determined by evaluating the phosphorylation of H2AX [a marker protein for the detection of DNA double-strand breaks (DSBs)], using flow cytometry. The combination of MK-1775 and C ions or X rays significantly increased H2AX phosphorylation at 24 h after irradiation, relative to radiation exposure alone (Fig. 6A), indicating suppression

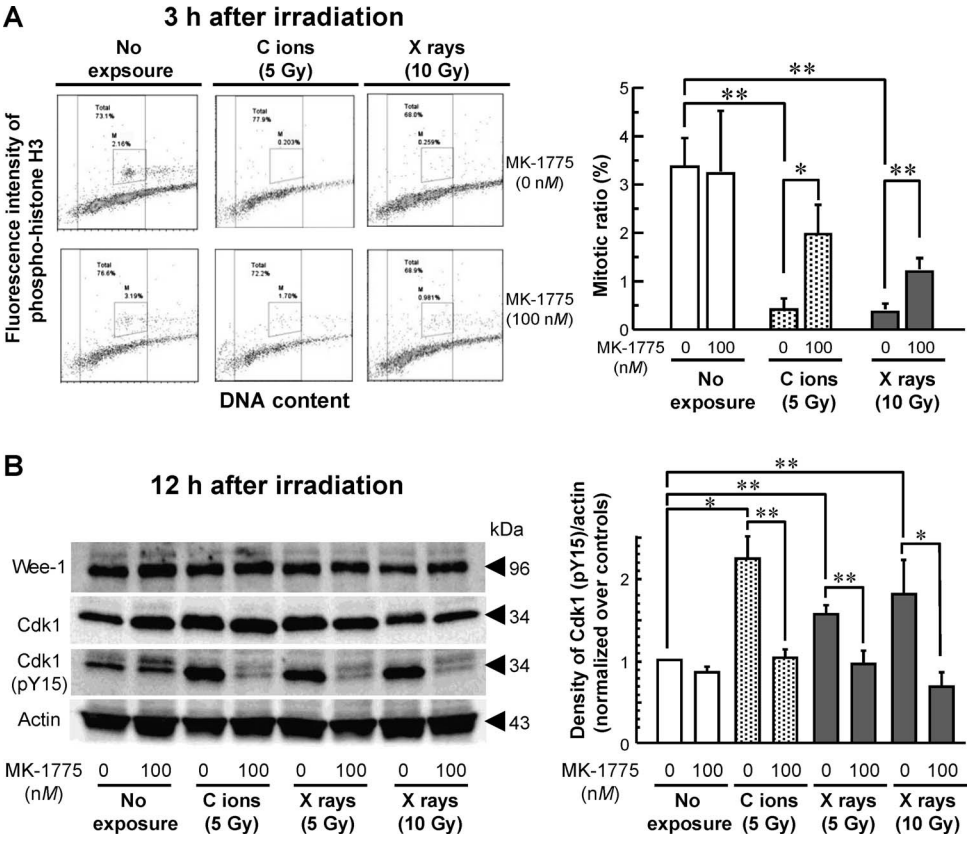


**FIG. 4.** Effect of MK-1775 and radiation on cell cycle regulation in *TP53*-null H1299 cells based on staining of phospho-histone H3 and cell cycle checkpoint-specific proteins. Panel A: Mitotic ratio of MK-1775-treated and/or irradiated cells. Panel B: Expression of Wee-1, accumulation of Cdk1, phospho-Cdk1 (Y15), and actin proteins in MK-1775- and/or radiation-treated cells and density of Cdk1 (Y15)/actin (analyzed using Scion image software). Results are presented as mean ± SD of three independent experiments. Data were analyzed using Student's *t* test (*$P < 0.05$; **$P < 0.01$).

of DNA repair ability and the presence of unrepaired, lethal DSB damage in the cancer cells. We also examined individual cells and followed mother and daughter cells up to 48 h after irradiation. Mitotic catastrophe was determined directly from time-lapse images as the percentage of multinuclear cells after abnormal mitosis postirradiation in the presence or absence of 100 n*M* MK-1775 or with MK-1775 alone. Mitotic catastrophe induced by either irradiation modality was significantly increased by the addition of MK-1775, compared with either treatment alone (Fig. 6B).

## DISCUSSION

The results of this study demonstrated that the Wee-1 inhibitor MK-1775 enhanced the sensitivities of human *TP53*-defective, *TP53*-mutated and *TP53*-proficient NSCLC cells to C ions, and may thus represent a useful strategy for improving the effectiveness of C-ion-based radiotherapy regimens. These finding are consistent with a previously reported study of photon irradiation of NSCLC cells (*10*). In the current study, radiation exposure increased the number of cells in $G_2$/M phase, with a peak at 12 h for both radiation modalities, independent of radiation dose. However, $G_2$ arrest induced by C ions was about 2.3 times stronger than that induced by X rays at the same physical dose, suggesting that C ions have greater biological effectiveness than X rays (*25, 26*) and induce more DNA damage. The effectiveness of C ions was further supported by the RBE of approximately 2.4, calculated from the surviving fraction. MK-1775 attenuated C-ion-induced $G_2$ arrest and restored the C-ion-induced reduction in mitotic ratio and increase in Cdk1 phosphorylation. Similar results using X rays have recently been reported in NSCLC and glioblastoma cell lines (*10, 23, 27*). The inhibitory effect of MK-1775 on C-ion-induced $G_2$ arrest at 250 n*M* reached saturation, in agreement with saturation of the SR at the same concentration.

Our results demonstrated that Wee-1 was expressed in H1299 cells (Fig. 4B). This was in agreement with the results of a recent study that showed Wee-1 overexpression in lung carcinoma (*16*), though another study found no Wee-1 expression in 65.8% of NSCLC (*28*). Wee-1 expression may therefore depend on pathological type or cell line. MK-1775 as a single agent has anticancer activity in NSCLC cells (*29*). Thus, although Wee-1 expression in

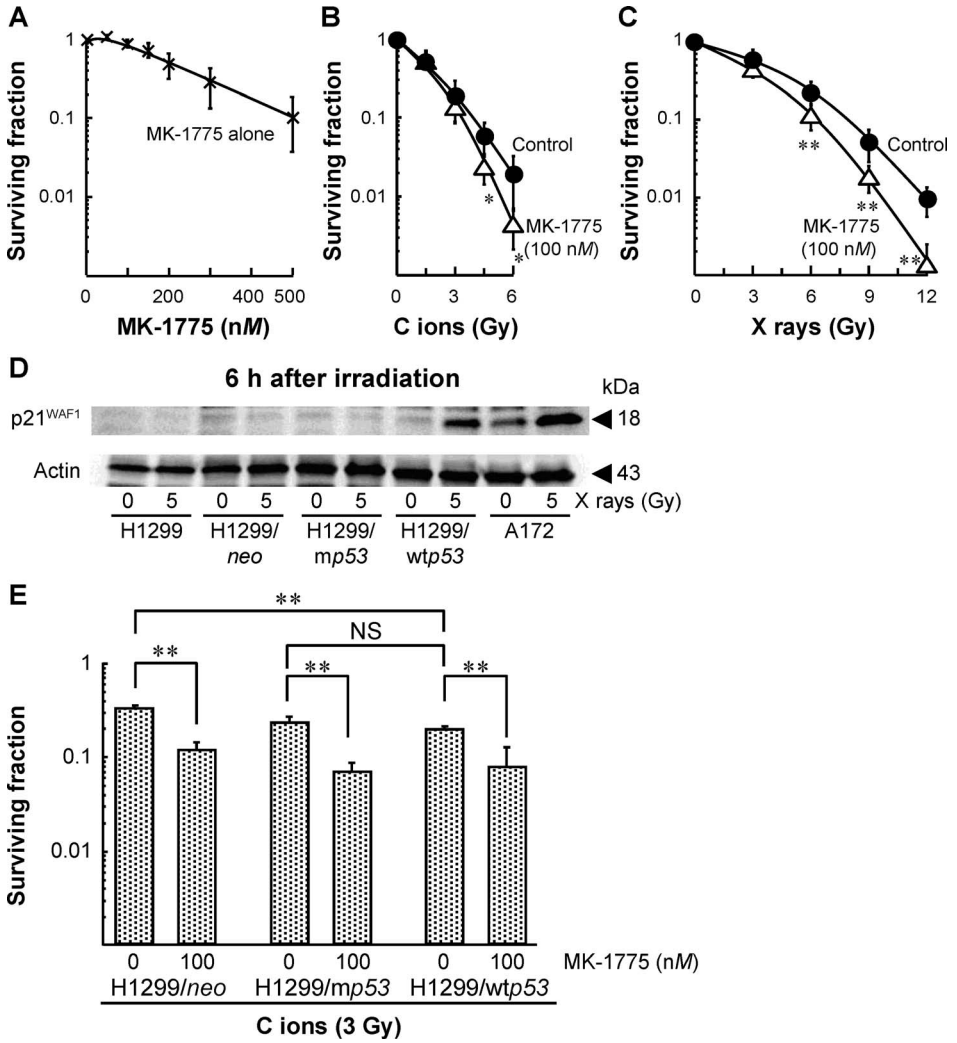


**FIG. 5.** Effect of MK-1775 and radiation on cell killing. MK-1775 concentration-dependent (panel A), C ion dose-dependent (panel B) and X-ray dose-dependent (panel C) changes in surviving fractions of H1299 cells. Panel D: Accumulation of $p21^{WAF1}$ and actin proteins in irradiated cells. Panel E: Surviving fractions of H1299/*neo*, H1299/m*p53* and H1299/wt*p53* cells after exposure to 3 Gy C ions in the presence or absence of 100 n*M* MK-1775. Results are presented as mean ± SD of three or four independent experiments. Data were analyzed using Student's *t* test (*$P < 0.05$; **$P < 0.01$; NS = nonsignificant).

various NSCLC cell lines has remained elusive, our results suggest that anti-Wee-1 therapy could be a potential treatment strategy for NSCLC (*30*).

In the presence of MK-1775, C ions had similar SR values to those of X rays, despite inducing more robust $G_2$ arrest than X rays. Furthermore, the sensitizing effect was additive rather than synergistic. This unexpected result may be explained by calculation of the SR at the dose that induced the same biological effects and the same level of $G_2$ arrest; the robust $G_2$ arrest induced by C ions thus occurred at the same physical dose. The cell cycle checkpoint is also known as the DNA damage checkpoint, and non-homologous end joining repair is a major DSB repair pathway (*31*) throughout the cell cycle (*32*). The additive effect of MK-1775 and C ions may thus be attributable to repair of C-ion-induced robust DSB damage, relative to X rays, by non-homologous end joining, after entry into abnormal M phase with persisting DNA damage. MK-1775 enhanced the sensitivity of *TP53*-null, m*p53* and wt*p53* cells to C ions in a *TP53*-independent manner. MK-1775 also sensitized *TP53*-proficient human lung cancer A549 cells. These results concur with those of previous studies in which the effects of MK-1775 alone or in combination with photon irradiation appeared to be *TP53*-independent in some NSCLCs, sarcomas and glioblastomas (*17, 23, 27, 29, 33*). In contrast, MK-1775 showed a *TP53*-dependent sensitizing effect, because only $G_2$ arrest is active in *TP53*-deficient cancer cells, while both $G_1$ and $G_2$ checkpoints are active in *TP53*-proficient cells (*10, 34, 35*). The reason for this difference is unclear but may be related to dysregulated $G_1$ arrest in wt*p53* cells or mutations in a gene functioning in *TP53* signaling (*36, 37*). *TP53* plays a critical role in the pathway controlling apoptosis, cell cycle checkpoint and DNA repair, and mutations in the

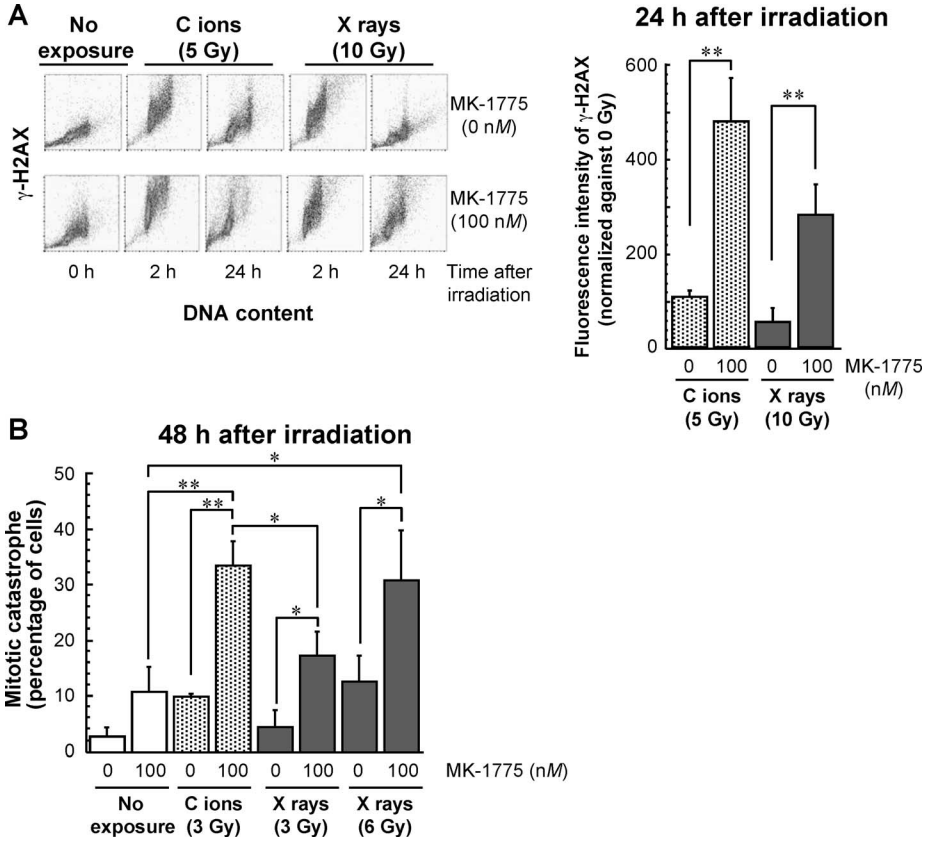


**FIG. 6.** Effect of MK-1775 and irradiation on DNA damage accumulation and cell death in *TP53*-null H1299 cells. Panel A: The fluorescence intensity of γ-H2AX in MK-1775 and irradiated cells. Panel B: Percentage of mitotic catastrophe in MK-1775 and irradiated cells at 48 h after irradiation. Results are shown as mean ± SD of three independent experiments. Data were analyzed using Student's *t* test (*$P < 0.05$; **$P < 0.01$).

*TP53* gene lead to radiation resistance (*38, 39*). Wee-1 checkpoint kinase is a potential target for synthetic lethality in *TP53*-deficient tumors (*39*). MK-1775 radiosensitized *TP53*-mutated NSCLC cells as well as *TP53*-proficient cells, suggesting that MK-1775 may be able to overcome this resistance at least to some degree.

This study was limited by the lack of measurements in normal cells, because normal lung cells are difficult to culture. Nevertheless, more cancer cells are in $G_2$ or M phase as a result of the higher proliferation ratio in cancer compared to normal cells, suggesting that MK-1775 has sufficient tumor specificity for clinical application, especially in therapies involving Wee-1-overexpressing cancers (*23*). In addition, as the main regulator of $G_1$ arrest, *TP53* is also a primary component of $G_2$ arrest (*40*). This suggests that normal cells with wild-type *TP53* have two $G_2$ checkpoint pathways, a *TP53*-dependent and a *TP53*-independent one, indicating that selective inhibition of $G_2$ arrest disrupting *TP53*-independent pathways should not harm normal cells (*12*) under a synthetic lethality mechanism.

Our study showed that the combination of MK-1775 and C ions or X rays significantly increased H2AX phosphorylation at 24 h postirradiation relative to irradiation alone. These results are comparable to some previous reports involving X rays (*10, 23, 27*), showing increased persistence of unrepaired DNA damage in MK-1775-pretreated cells.

The combination of MK-1775 and C ions or X rays also significantly increased mitotic catastrophe compared to either treatment alone. These results confirm those of previous reports in which the combination of MK-1775 and DNA-damaging cancer therapy induced a significant increase in mitotic catastrophe (*16, 23*), and in which C ions killed X-ray-resistant *TP53*-null cancer cells by inducing mitotic catastrophe (*20*). In a recent study, Wee-1 inhibition was reported to reverse the defective apoptosis of tumor cells by forced Cdk1 activity, resulting in alleviation of resistance to immune attack by cytotoxic T lymphocytes, natural killer cells and lymphokine-activated killer cells (*41*). The immune surveillance system imposes immunoselection to eliminate abnormal mitosis-induced hyperploid cells in oncogene- and carcinogen-induced cancers (*42*), indicating that cell cycle $G_2$ checkpoint inhibitor-induced hyperploid cancer cells may be eliminated by immune attack through immunosurveillance. Our study highlights the utility of MK-1775 for enhancing the sensitivity of *TP53*-defective and *TP53*-proficient NSCLC cells to C-ion irradiation, and for targeting Wee-1 to improve the outcome of heavy-ion beam cancer therapy.

In conclusion, the cell cycle regulation inhibitor MK-1775 was found to enhance the radiosensitivity of human *TP53*-defective, *TP53*-mutated and *TP53*-proficient NSCLC cells to C ions and X rays by abrogating the Wee-1 phospho-Cdk1-mediated $G_2$ checkpoint after irradiation. MK-1775 induced $G_2$-phase cells to enter mitosis prematurely and progress into the next cell cycle with incompletely repaired DNA damage, eventually causing the accumulation of DSBs and mitotic catastrophe. This targeting of cell cycle checkpoints offers a strategy for the treatment of NSCLC and for improving existing C-ion-based radiotherapy regimens.

## SUPPLEMENTARY INFORMATION

**Fig. S1.** Effect of MK-1775 and irradiation on cell killing of *TP53*-proficient human lung cancer A549 cells.

## ACKNOWLEDGMENTS

This work was supported by the Program to Disseminate the Tenure Tracking System and Program for Cultivating Global Leaders in Heavy Ion Therapeutics and Engineering of the Ministry of Education, Culture, Sports, Science and Technology of Japan granted to Gunma University. The study was performed as part of the Research Project with Heavy Ions at the Gunma University Heavy Ion Medical Center.

Received: June 15, 2015; accepted: September 30, 2015; published online: December 5, 2015

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# ERRATA

Volume **184**, Number 6 (2015); pp. 660–669, in the article “Targeting of Carbon Ion-Induced $G_2$ Checkpoint Activation in Lung Cancer Cells Using Wee-1 Inhibitor MK-1775” by Hongyu Ma *et al*.

Page 661: Radiosensitivity Assays, line 5:

“after 9 days” should be “after 7–9 days”.

Page 661: the first and second formulas should read as:

RBE = $D_{10}$ of X-irradiated cells / $D_{10}$ of C ion-irradiated cells

Page 668: Reference 16:

“*16. 16*. De” should be “*16*. De”.

Page 668: Reference 17:

“*17*. De Witt Hamer PC, Mir SE” should be “*17*. Mir SE, De Witt Hamer PC”.

Page 669: Reference 38:

“2008; 50:37–42.” should be “2009; 50:37–42.”.

Page 669: Reference 39:

“Tr Cell Bio 2015; 25:8.” should be “Trends Cell Biol 2015; 25:486–95.”.