In vitro and In vivo Radiosensitization with AZD6244 (ARRY-142886), an Inhibitor of Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase 1/2 Kinase

Eun Joo Chung,1 Aaron P. Brown,2 Hiroaki Asano,1 Mariana Mandler,1 William E. Burgan,3 Donna Carter,3 Kevin Camphausen,1 and Deborah Citrin1

Abstract Purpose: The mitogen-activated protein (MAP) kinase pathway is important for cell proliferation, survival, and differentiation, and is frequently up-regulated in cancers. The MAP kinase pathway is also activated after exposure to ionizing radiation. We investigated the effects of AZD6244 (ARRY-142886), an inhibitor of MAP kinase/extracellular signal-regulated kinase 1/2, on radiation response.

Experimental Design: The effects of AZD6244 on the in vitro radiosensitivity of human cancer cell lines (A549, MiaPaCa2, and DU145) were evaluated using clonogenic assays. DNA damage repair was evaluated using γH2AX, and mitotic catastrophe was measured using nuclear fragmentation. Cell cycle effects were measured with flow cytometry. Growth delay was used to evaluate the effects of AZD6244 on in vivo tumor radiosensitivity.

Results: Exposure of each cell line to AZD6244 before irradiation resulted in an increase in radiosensitivity with dose enhancement factors at a surviving fraction of 0.1, ranging from 1.16 to 2.0. No effects of AZD6244 on radiation-induced apoptosis or persistence of γH2AX foci after irradiation were detected. Cells treated with AZD6244 had an increased mitotic index and decreased Chk1 phosphorylation at 1 and 2 hours after irradiation. Mitotic catastrophe was increased in cells receiving AZD6244 and irradiation compared with the single treatments. In vivo studies revealed that AZD6244 administration to mice bearing A549 tumor xenografts resulted in a greater than additive increase in radiation-induced tumor growth delay (dose enhancement factor of 3.38).

Conclusions: These results indicate that AZD6244 can enhance tumor cell radiosensitivity in vitro and in vivo and suggest that this effect involves an increase in mitotic catastrophe.

The mitogen-activated protein (MAP) kinase cascades play an important role in the progression and maintenance of cancer. The extracellular signal-regulated kinase (ERK) MAP kinase cascade is known to be involved in cell proliferation, cell survival, and metastasis. Inhibition of the ERK MAP kinase pathway may allow inhibition of signaling through multiple upstream receptors and intermediates such as epidermal growth factor receptor, Ras, and Raf, which are frequently mutated, up-regulated or constitutively active in cancers.

Activation of the Raf–MAP/ERK kinase (MEK)–ERK pathway occurs rapidly in tumor cells after exposure to ionizing radiation (1–3). Activation of the Ras-Raf-MEK-ERK cascade, although mutations in Ras and Raf is known to result in enhanced tumor cell proliferation and enhanced survival after irradiation (4–6). Furthermore, inhibition of Ras and Raf in cell lines with activating Ras mutations results in sensitization to ionizing radiation (7–9). These data suggest that inhibition of the Ras-Raf-MEK-ERK cascade may sensitize cells to ionizing radiation.

AZD6244 is a novel, selective, ATP-competitive inhibitor of MEK1/2 (10). AZD6244 has been reported to inhibit tumor growth via inhibition of MEK1/2 signaling and as a consequence through inhibition of regulators of cell proliferation and the cell cycle, including cyclin D1, cdc-2, cyclin-dependent kinases 2 and 4, cyclin B1, and c-Myc (11). AZD6244 has broad preclinical activity against several tumor histologies in cell-based growth assays and in mouse xenograft models, including melanoma (12), non–small cell lung (13), colorectal (10, 13), pancreatic (10), and hepatocellular carcinomas (11). AZD6244 is a clinically relevant molecule; a phase I trial of AZD6244 as a single agent resulted in a high rate of disease stabilization in patients with solid tumors, with rash representing the most common toxicity (14). Complete and partial responses to AZD6244 have been seen in phase II monotherapy trials in patients with advanced cancer (15, 16).
To pursue MEK inhibition as an approach to radiosensitize tumor cells, we have investigated the effects of treatment with AZD6244 of the radiosensitivity of three human tumor cell lines of different histologies. The data presented indicate that AZD6244 enhanced the \textit{in vitro} sensitivity of each cell line to irradiation. Sensitization \textit{in vitro} was accompanied by an increase in the percentage of treated cells dying by mitotic catastrophe. Lastly, xenograft studies showed that AZD6244 administration before irradiation results in a greater than additive increase in tumor regrowth delay in a dose-dependent fashion.

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\begin{table}[h]  
\centering  
\begin{tabular}{|c|c|c|}  
\hline  
Cell line & Treatment & Result \hline
MiaPaCa2 & Control & \hline
MiaPaCa2 & AZD6244 & \hline
MiaPaCa2 & AZD6244 + Irradiation & \hline
\hline  
DU1145 & Control & \hline
DU1145 & AZD6244 & \hline
DU1145 & AZD6244 + Irradiation & \hline
\hline  
A549 & Control & \hline
A549 & AZD6244 & \hline
A549 & AZD6244 + Irradiation & \hline
\hline  
\end{tabular}  
\caption{Summary of results for different cell lines treated with AZD6244 alone or in combination with irradiation.}  
\end{table}

Materials and Methods

\textbf{Cell lines and treatment.} The MiaPaCa2 (pancreatic adenocarcinoma), DU1145 (prostatic adenocarcinoma), and A549 (non–small cell lung cancer) cell lines were obtained from the Division of Cancer Treatment and Diagnosis Tumor Repository, NCI-Frederick (Frederick, Maryland). Cells were cultured in RPMI 1640 medium (Quality Biological) containing 2 mmol/L glutamine supplemented with 5% CO$_2$. MiaPaCa2, provided by Astra Zeneca, reconstituted in DMSO, and stored at -20°C. Cultures were irradiated using a Pantak X-ray source at a dose rate of 1.5 Gy/min.

\textbf{Clonogenic assay.} Cells were trypsinized to generate a single cell suspension, and a specified number of cells were seeded into each well of six-well tissue culture plates. After allowing 6 h for attachment, the cells were incubated with AZD6244 (100 nmol/L for MiaPaCa2 and 250 nmol/L for A549 and DU1145) or DMSO (vehicle control) for 16 h before irradiation. Twelve to 14 d after seeding, colonies were stained with crystal violet, the number of colonies containing at least 50 cells was determined, and the surviving fractions were calculated. Survival curves were generated after normalizing for cytotoxicity generated by AZD6244 alone for each independent experiment. Data presented are the mean ± SE from at least three independent experiments.

\textbf{Cell cycle analysis.} To assess cell cycle distribution, cells were treated as described in the clonogenic survival assays, except that each cell was seeded in 100 mm dishes. Cells were harvested by trypsinization at each indicated time point, rinsed with cold PBS, and fixed with 70% ice-cold ethanol overnight at 4°C. Fixed cells were rinsed with cold PBS followed by incubation with PBS containing 10 μg/ml propidium iodide and 0.5 mg/ml RNase A for 15 min at 37°C. The DNA content of labeled cells was acquired using FACScan Caliber cytometry (BD Biosciences) and FlowJo software (Tree Star, Inc.).

\textbf{Apoptotic cell death.} The Guava Nexin assay (part number 4500-0161) was done following the manufacturer’s instructions. Briefly, 3 × 10$^4$ cells (50 μL) were added to a 150 μL staining solution containing 135 μL of apoptosis buffer, 10 μL Annexin V-PE, and 5 μL of 7-AAD. The cells were incubated in the dark at room temperature for 20 min. Samples (2,000 cells/well) were then acquired on the Guava EasyCyte system.

\textbf{Immunofluorescent staining for γH2AX.} Cells grown in tissue culture chamber slides were fixed with 1% paraformaldehyde, permeabilized with 0.4% Triton X-100, and blocked with 2% bovine serum albumin in PBS. The cells were stained with anti-γH2AX antibody (Millipore Corp.), washed, and incubated with fluorescence conjugated secondary antibodies (Molecular Probes/Invitrogen) and 4',6-diamidino-2-phenylindole (Sigma-Aldrich). Slides were examined on a Leica DMRXA fluorescent microscope. Images were captured by a Photometrics Sensys CCD camera (Roper Scientific) and imported into IP Labs image analysis software package (Scanalytics, Inc.). For each treatment condition, the total number of γH2AX foci per cell was determined in at least 150 cells.

\textbf{Mitotic catastrophe.} The presence of fragmented nuclei was used as the criteria for defining cells undergoing mitotic catastrophe. To visualize nuclear fragmentation, cells were fixed with methanol for 15 min at -20°C, stained with anti–α-tubulin monoclonal antibody (Sigma-Aldrich; T6199) followed by staining with FITC-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc.). Nuclei were counterstained with 4',6-diamidino-2-phenylindole. A total of 150 randomly selected cells were analyzed for each treatment group and photographed with epi-fluorescence. Nuclear fragmentation was defined as the presence of more than two distinct nuclear lobes within a single cell.

\textbf{In vivo tumor model.} Four to 6-wk-old female nude mice (Fredrick Labs) were used in these studies. Mice were caged in groups of five or less, and all animals were fed a diet of animal chow and water \textit{ad libitum}. Tumor cells (5 × 10$^5$ cells) were injected s.c. into the right hind leg.

When tumors grew to a mean volume of 172 mm$^3$, the mice were randomized to vehicle alone, AZD6244 alone, AZD6244 plus irradiation (RT), or RT alone. The mice were given a single oral dose of AZD6244 at 50 mg/kg. Four hours after drug administration, the mice received a dose of 3 Gy to the tumor. Irradiation was done using a Pantak irradiator, with animals restrained in a custom jig. To obtain a tumor growth curve, perpendicular diameter measurements of each tumor were measured every 3 d with a digital calipers, and volumes were calculated using a formula (length \times width \times height)/2. Tumors were followed until the tumors of the group reached a mean size of 1,500 mm$^3$. Specific tumor growth delay was calculated for each individual animal. The mean growth delay for each treatment group was calculated as the number of days for the mean of the treated tumors to grow to 1,500 mm$^3$ minus the number of days for the mean of the control group to reach the same size. SD in days were calculated about the mean of the treated groups. Each experimental group contained five mice. The control group contained 10 mice. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals.

\textbf{Western blotting.} Cell extracts were prepared using radioimmuno-precipitation assay buffer (Pierce) containing protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma-Aldrich), followed by measurement of protein concentrations by the Bradford method (Bio-Rad). Equal amounts of protein were subjected to Western blot analysis, which were probed with the primary antibody indicated. ImageQuant software was used to evaluate the relative expression of phosphorylated ERK1/2 and total ERK1/2 normalized to actin, the loading control in Western blots of three cell lines.

\textbf{Statistical analysis.} \textit{In vitro} experiments were repeated thrice, and statistical analysis was done using a Student’s $t$ test. Data are presented as mean ± SD. A probability level of $P < 0.05$ was considered significant.

\textbf{Results.} To determine the effects of AZD6244 on tumor cell radiosensitivity, clonogenic survival analysis was done in the A549, MiaPaCa2, and DU1145 cell lines. The AZD6244 concentration...
selected for each cell line was based on toxicity studies such that the dose resulted in ~50% toxicity as a single agent (100 nmol/L for MiaPaCa2 and 250 nmol/L for A549 and DU145). As shown in Fig. 1, AZD6244 treatment delivered 16 hours before irradiation increased A549, DU145, and MiaPaCa2 radiosensitivity with a dose enhancement factor at a surviving fraction of 0.10 of 2.0, 1.36, and 1.16, respectively.

To confirm target activation after irradiation, we evaluated phosphorylation of ERK1/2, a signaling intermediate immediately downstream of MEK1/2 in the A549, MiaPaCa2, and DU145 cell lines. Radiation-induced ERK1/2 phosphorylation was evident 2 hours after irradiation. In conditions used for clonogenic assays, AZD6244 decreased radiation-induced ERK1/2 phosphorylation in the A549, MiaPaCa2, and DU145 cell lines (Fig. 2). Thus, at the dose of AZD6244 used to enhance the response to radiation, there is an inhibition of phosphorylation of ERK1/2 after irradiation.

To further investigate the cellular processes through which AZD6244 enhances radiosensitivity, we focused on the A549 and MiaPaCa2 cell lines. DNA damage repair is an important component of radiation-induced cytotoxicity. As a measure of radiation-induced DNA damage, we evaluated induction of nuclear foci of phosphorylated histone H2AX (γH2AX), which has been established as a sensitive indicator of DNA double-strand breaks with the resolution of foci corresponding to double-strand break repair (17). Cells were exposed to AZD6244 for 16 hours and irradiated (4 Gy) as in the cell survival experiments, and γH2AX foci were determined at 1, 6, and 24 hours postirradiation. Exposure of cells to AZD6244 only for 16 hours resulted in no significant increase in the number of γH2AX foci in the A549 and MiaPaCa2 cell lines (Supplemental Fig. S1). Irradiation (4 Gy) only induced a significant increase in the number of γH2AX foci at 1 hour, which progressively declined to 24 hours. Exposure to AZD6244 followed by 4 Gy resulted in a number of γH2AX foci not significantly different to that observed with RT alone at 1 hour; thus, AZD6244 does not impact the immediate DNA damage after irradiation. At 24 hours, the number of γH2AX foci per cell was similar in the irradiation and combination group; thus, AZD6244 does not inhibit DNA double-strand break repair.

Cell cycle analysis after pretreatment with AZD6244 revealed no evidence of redistribution into radiosensitive phases of the cell cycle (Fig. 3A). Treatment with AZD6244 resulted in a lower percentage of cells in the G2/M phase of the cell cycle compared with cells treated with vehicle alone. Another potential source of radiosensitization is the abrogation of the G2 checkpoint, which is considered to protect against radiation-induced cell death (18). Flow cytometric analysis of phosphorylated histone H3 in the 4n cell population at several time points after irradiation was used to distinguish cells in G2 and M phases of the cell cycle. This assay provides a measure of the progression of G2 cells into M phase and thus the activation of the G2 checkpoint (19). As shown in Fig. 3B, irradiation (4 Gy) resulted in a rapid reduction in the mitotic index reaching a maximum decrease at 3 hours, indicating activation of the early G2 checkpoint. AZD6244 treatment prevented the decrease in the mitotic index after irradiation, suggesting that AZD6244 treatment abrogated the early G2 checkpoint. No difference in the mitotic index was appreciated in A549 cells at 24 and 48 hours after irradiation with 4 Gy.

The Chk1 pathway is known to be involved in activation of the G2 checkpoint and in radiation response (18, 20). We observed an abrogation of the G2 checkpoint after irradiation in cells treated with AZD6244. Therefore, we evaluated phosphorylation of Chk1 in irradiated cells treated with vehicle control or AZD6244. Treatment with AZD6244 resulted in impaired Chk1 phosphorylation after irradiation compared with that observed in vehicle-treated cells (Fig. 4). In addition, treatment with AZD6244 reduced the expression of total Chk1 protein in unirradiated cells compared with that in vehicle-treated unirradiated cells.

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**Fig. 1.** The effects of AZD6244 on tumor cell radiosensitivity. Cell lines A549 (A), MiaPaCa2 (B), and DU145 (C) were exposed to AZD6244 (250 nmol/L for A549 and DU145, 100 nmol/L for MiaPaCa2) or vehicle control for 16 h and irradiated with graded doses of X-rays. Colony-forming efficiency was determined 10 to 14 d later and survival curves generated after normalizing for cell killing by AZD6244 alone. The mean of three independent experiments. PE, plating efficiency with AZD6244; DEF, dose enhancement factor. Points, mean; bars, ±SE.
Davies et al. (13) reported an increase of activated caspase-3, one of the principal effectors of apoptosis in a xenograft model after treatment with AZD6244. To define the contribution of apoptosis to the AZD6244-mediated radiosensitization of cancer cells, membrane alterations in early phase of apoptosis were determined in cells at 24, 48, and 72 hours after irradiation (4 Gy). As shown in Fig. 5A and B, there was a nonsignificant increase in apoptosis with radiation and treatment with AZD6244 compared with untreated controls; however, the degree of apoptosis that was measured when combining AZD6244 and RT was less than additive in the A549 and MiaPaCa2 cell lines. Thus, the combination of AZD6244 and RT shown to enhance radiation-induced death in Fig. 1 had no effect on the frequency of apoptotic cell death. These data indicate that the AZD6244-mediated radiosensitization of A549 cells does not involve significantly enhanced susceptibility to apoptosis.

The observation that cells treated with AZD6244 did not arrest in G2 after irradiation suggests that mitotic catastrophe may be a mechanism of increased cell death after treatment with AZD6244 and irradiation. To test if mitotic catastrophe could be responsible for decreased clonogenic survival in A549 cells treated with AZD6244 and RT, the number of cells with abnormal nuclei as a function of time after irradiation was scored (21). Cells undergoing mitotic catastrophe could be clearly distinguished after the individual treatment of irradiation (4 Gy) and AZD6244 (as described in Methods and Materials), as well as the combination. As shown in Fig. 5C and D, there was a time-dependent increase in the number of cells undergoing mitotic catastrophe after the individual treatments with radiation and AZD6244 out to at least 96 hours. In cells receiving the combination treatment, a significant increase in the percentage of cells undergoing mitotic catastrophe was detected at 72 hours posttreatment in the A549 and MiaPaCa2 cell lines. This finding was accompanied by an increase in the proportion of cells containing >4n DNA content by flow cytometry (Supplemental Fig. S2). An increase in cells containing >4n DNA was detected within 24 hours after radiation in cell lines treated with vehicle or AZD6244. In addition, cells containing >4n DNA were significantly increased in A549 and MiaPaCa2 cells treated with AZD6244 compared with those treated with vehicle alone 96 hours after irradiation. These data thus suggest that the AZD6244-mediated radiosensitization is mediated by the failure of recovery after irradiation resulting in an increase in the cells undergoing mitotic catastrophe.

To determine whether the enhancement of tumor cell radiosensitivity measured in vitro could be translated into an in vivo tumor model, a tumor growth delay assay using A549 and MiaPaCa2 cells grown s.c. in the hind leg of nude mice was used. Mice bearing sc xenografts (172 mm3) were randomized into four groups: vehicle; AZD6244 only (50 mg/kg); irradiation (3 Gy) only; and AZD6244 (50 mg/kg) administered by oral gavage 4 hours before irradiation (3 Gy). Treatment was on the
day of randomization. The growth rates for the A549 and MiaPaCa2 tumors exposed to each treatment are shown in Fig. 6A and B, respectively. For each group, the time to grow from 172 mm$^3$ (volume at the time of treatment) to 1,500 mm$^3$ was calculated using the tumor volumes from the individual mice in each group (mean ± SE).

For the A549 xenograft model, the time required for tumors to grow from 172 to 1,500 mm$^3$ increased from 24.8 ± 1.0 days for vehicle-treated mice to 40.0 ± 1.7 days for AZD6244 (50 mg/kg)–treated mice. Irradiation treatment alone increased the time to reach 1,500 mm$^3$ to 35.6 ± 1.5 days. However, in mice that received the AZD6244 + irradiation combination, the time for tumors to grow to 1,500 mm$^3$ increased to 61.4 ± 1.9 days (50 mg/kg AZD6244). The absolute growth delays (the time in days for tumors in treated mice to grow from 172 to 1,500 mm$^3$ minus the time in days for tumors to reach the same size in vehicle treated mice) were 15.2 for 50 mg/kg AZD6244 alone and 10.8 for irradiation alone; the tumor growth delay induced by the AZD6244 + irradiation treatment was 36.6 (50 mg/kg AZD6244). Thus, the growth delay after the combined treatment was more than the sum of the growth delays caused by individual treatments. To obtain a dose enhancement factor comparing the tumor radiation response in mice with and without AZD6244 treatment, the normalized tumor growth delays were calculated, which account for the contribution of AZD6244 to tumor growth delay induced by the combination treatment. Normalized tumor growth delay was defined as the time in days for tumors to grow from 172 to 1,500 mm$^3$ in mice exposed to the combined modality minus the time in days for tumors to grow from 172 to 1,500 mm$^3$ in mice treated with AZD6244 only. The dose enhancement factor, obtained by dividing the normalized tumor growth delay in mice treated with AZD6244 + irradiation by the absolute growth delay in mice treated with radiation only, was 3.38 for 50 mg/kg of AZD6244.

A similar experiment was done in MiaPaCa2 xenografts. The growth rates for the MiaPaCa2 tumors exposed to each treatment are shown in Fig. 6B. For the MiaPaCa2 xenograft model, the time required for tumors to grow from 172 to 1,500 mm$^3$ increased from 35.8 ± 1.4 days for vehicle-treated mice to 44.4 ± 1.8 days for AZD6244 (50 mg/kg)–treated mice. Irradiation treatment alone increased the time to reach 1,500 mm$^3$ to 41.8 ± 2.3 days. However, in mice that received the AZD6244 + irradiation combination, the time for tumors to grow to 1,500 mm$^3$ increased to 54.8 ± 1.2 days (50 mg/kg AZD6244). The absolute growth delays (the time in days for tumors in treated mice to grow from 172 to 1,500 mm$^3$ minus the time in days for tumors to reach the same size in vehicle-treated mice) were 8.5 for 50 mg/kg AZD6244 alone and 5.9
for irradiation alone; the tumor growth delay induced by the AZD6244 + irradiation treatment was 18.9 (50 mg/kg AZD6244). Thus, the growth delay after the combined treatment was more than the sum of the growth delays caused by individual treatments. The dose enhancement factor for the addition of AZD6244 in the MiaPaCa2 xenograft model was 2.3.

These data indicate that AZD6244 significantly enhances the radiation-induced cytotoxicity in vitro in clonogenic assays and in a tumor growth delay in A549 and MiaPaCa2 xenografts. These effects correlate to a decrease in activation of the G2 checkpoint and an increase in mitotic catastrophe after irradiation in AZD6244-treated cells compared with cells treated with irradiation alone.

Discussion

An understanding of signal transduction events occurring after irradiation and the development of inhibitors of these pathways has opened new avenues of research into the use of targeted therapies as radiation sensitizers. Signaling through the Ras-Raf-MEK-ERK pathway is known to be important in radiation response and radiation resistance (4–6). Therefore, inhibition of this pathway may be an attractive means to sensitize tumor cells to ionizing radiation. The availability of AZD6244, a specific inhibitor of MEK1/2, provides a means to test this hypothesis with a clinically relevant molecule (10).

The data presented here indicate that AZD6244 enhances the radiosensitivity of tumor cells in vitro and in vivo. Treatment of the A549, MiaPaCa2, and DU145 cell lines with AZD6244 resulted in an increase in radiation response. Treatment of these same cell lines with AZD6244 with the same concentration used in clonogenic assays resulted in inhibition of ERK1/2 activation, a specific target of AZD6244 and a downstream signaling event following irradiation.

Most cell lines sensitive to AZD6244 as a single agent (IC50, <1 μmol/L) have been found to possess activating mutations in BRAF, KRAS and NRAS genes (13). The two KRAS mutant cell lines that were tested, A549 and MiaPaCa2, exhibited greater sensitization to radiation when treated with AZD6244 compared with the RAS wild-type line, DU145. The DU145 cell line is known to express epidermal growth factor receptor and secrete epidermal growth factor, which acts via an autocrine method to stimulate growth (22). Inhibition of epidermal growth factor receptor has been shown to enhance radiation

![Fig. 5. The effects of AZD6244 on the mechanism of cell death after irradiation.](www.aacrjournals.org)
response in a variety of cell lines, including the DU1145 cell line (23). It is possible that inhibition of this autocrine signaling pathway with AZD6244 treatment contributed to the observed increase in radiation sensitivity.

The finding that the two KRAS mutant lines were preferentially sensitized is hypothesis generating, given that three lines were tested. Additional work will be needed to clarify if cell lines harboring KRAS mutations exhibit greater sensitization to radiation with AZD6244 treatment compared with RAS wild-type lines. This information would important implications for eventual clinical translation of AZD6244 as a radiation sensitizer. Additional work will be required to determine what molecular characteristics predict for enhanced radiation re-sensitizer. Further studies will focus on molecular characterization of cells undergoing mitotic catastrophe after irradiation in AZD6244-treated cells compared with vehicle controls.

Because AZD6244 treatment has been associated with alterations in modifiers of the cell cycle (12, 24, 25), we evaluated whether cell cycle effects could explain the observed increase in radiation response in the presence of AZD6244. Pretreatment of cells with AZD6244 as in clonogenic assays did not redistribute cells into the radiosensitive G2 and M phases of the cell cycle, suggesting that reassortment into a sensitive phase of the cell cycle was not the mechanism responsible for increased radiation response.

In contrast, postirradiation cell cycle analysis revealed that treatment of cells with AZD6244 resulted in an increase in the mitotic index compared with vehicle-treated cells, suggesting that AZD6244-treated cells had an impaired activation of the G2/M checkpoint after irradiation. Activation of the G2 checkpoint is considered protective from radiation-induced cell death (26).

In support of the observation that AZD6244 treatment inhibited G2 checkpoint activation after irradiation, ERK1/2 activation is required for carcinoma cells to arrest in at the G2 checkpoint via Chk1 pathway (27, 28). We found that AZD6244 treatment before irradiation led to a reduction in phospho-Chk1, likely a contributor to the abrogated G2 checkpoint.

Prolonged G2 arrest after genotoxic stress allows DNA damage repair before progression through mitosis (29, 30). Although we observed an early increase in the mitotic index in AZD6244-treated cells compared with controls, we did not observe significant differences in the number of γH2AX foci after significant irradiation. This suggests that radiation-induced DNA damage was repaired at similar rates in AZD6244 and vehicle-treated cells. Importantly, AZD6244 inhibited only the early G2 arrest after irradiation in AZD6244-treated cells as evidenced by an increased mitotic index as early as 1 hour after irradiation with a similar mitotic index to vehicle-treated cells at 24 hours. Many cells treated with irradiation and AZD6244 or vehicle control had elevated γH2AX foci at 1 and 6 hours compared with unirradiated controls. This suggests that treatment with AZD6244 allowed progression of cells with unrepaired DNA damage through the G2 checkpoint but did not inhibit DNA repair.

Cells that escape the initial G2 checkpoint delay after irradiation may continue through mitosis with incomplete cytokinesis with cell death or continued progression through the cell cycle (31) with eventual death by mitotic catastrophe (32). Inhibition of Chk1 after exposure to ionizing radiation results in an increased incidence of mitotic catastrophe and an impaired activation of cell cycle checkpoints (33). This is consistent with our observation of increased rates of mitotic catastrophe after irradiation in AZD6244-treated cells compared with vehicle controls.

In summary, we show that inhibition of the Ras-Raf-MEK-ERK signaling pathway with AZD6244 enhances radiation response in vitro and in vivo. This effect correlates to an abrogation in the G2 checkpoint and an increase in the number of cells undergoing mitotic catastrophe after irradiation in the presence of AZD6244. Future studies will focus on molecular characteristics that may predict the extent of sensitization such as the presence or absence of KRAS mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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