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In vitro and in vivo radiosensitization with AZD6244 (ARRY-142886), an inhibitor of Mitogen Activated Protein Kinase/ Extracellular Signal-regulated Kinase 1/2 kinase

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Abstract

Purpose—The Mitogen-activated Protein Kinase (MAPK) pathway is important for cell proliferation, survival and differentiation and is frequently upregulated in cancers. The MAPK pathway is also activated after exposure to ionizing radiation. We investigated the effects of AZD6244 (ARRY-142886), an inhibitor of MEK1/2, on radiation response.

Experimental Design—The effects of AZD6244 on the *in vitro* radiosensitivity of human cancer cell lines (A549, MiaPaCa2 and DU145) was evaluated using clonogenic assays. DNA damage repair was evaluated using γ H2AX 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 prior to irradiation (IR) resulted in an increase in radiosensitivity with dose enhancement factors (DEF) 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 γ H2AX foci after IR were detected. Cells treated with AZD6244 had an increased mitotic index and decreased Chk1 phosphorylation at 1 and 3 hours after IR. Mitotic catastrophe was increased in cells receiving both AZD6244 and IR compared to 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 (DEF 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.

INTRODUCTION

The mitogen-activated protein kinase (MAPK) cascades play an important role in the progression and maintenance of cancer. The ERK MAPK cascade is known to be involved in cell proliferation, cell survival, and metastasis. Inhibition of the ERK MAPK pathway may allow inhibition of signaling through multiple upstream receptors and intermediates such as

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EGFR, Ras, and Raf which are frequently mutated, upregulated, or constitutively active in cancers.

Activation of the Raf-MEK-ERK pathway occurs rapidly in tumor cells after exposure to ionizing radiation (1–3). Activation of the Ras-Raf-MEK-ERK cascade through 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, adenosine triphosphate–uncompetitive 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 on the radiosensitivity of three human tumor cell lines of different histologies. The data presented indicate that AZD6244 enhanced the *in vitro* sensitivity of each cell line to irradiation. Sensitization *in vitro* was accompanied by an increase in the percentage of treated cells dying by mitotic catastrophe. Lastly, xenograft studies showed that AZD6244 administration prior to irradiation results in a greater than additive increase in tumor regrowth delay in a dose-dependent fashion.

METHODS AND MATERIALS

Cell Lines and Treatment

The MiaPaCa2 (pancreatic adenocarcinoma), DU145 (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, Gaithersburg, Maryland) containing 2 mM L-glutamine, supplemented with 5% fetal bovine serum (Hyclone, Logan, Utah). Cells were maintained at 37°C, 5% CO₂. AZD6244, provided by Astra Zeneca, was reconstituted in DMSO and stored at –20°C. Cultures were irradiated using a Pantak (Solon, OH) X-ray source at a dose rate of 1.55 Gy/min.

Clonogenic Assay

Cell cultures 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 hours for attachment, the cells were incubated with AZD6244 (100 nM for MiaPaCa2, and 250 nM for A549 and DU145) or DMSO (vehicle control) for 16 hours prior to irradiation. Twelve to 14 days 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 ± SEM from at least three independent experiments.

Cell Cycle Analysis

To assess cell cycle distribution, cells were treated as described in the clonogenic survival assays, except that cells were 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 minutes at 37°C. The DNA content of labeled cells was acquired using FACSCaliber cytometry (BD Biosciences, San Jose, USA) and FlowJo software (Tree Star Inc., Ashland, OR).

Apoptotic Cell Death

The Guava Nexin assay (Part Number 4500-0161) was performed 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 minutes. Samples (2000 cells/well) were then acquired on the Guava EasyCyte system.

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 (BSA) in PBS. The cells were stained with anti-γH2AX antibody (Millipore Corp.), washed, and incubated with fluorescence conjugated secondary antibodies (Molecular Probes/Invitrogen,) and DAPI (Sigma-Aldrich, St. Louis, MO). Slides were examined on a Leica DMRXA fluorescent microscope (Wetzlar, Germany). Images were captured by a Photometrics Sensys CCD camera (Roper Scientific, Tucson, AZ) and imported into IP Labs image analysis software package (Scanalytics, Inc., Fairfax, VA). For each treatment condition, the total number of γH2AX foci per cell was determined in at least 150 cells.

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 minutes at -20°, stained with anti-α-tubulin monoclonal antibody (Sigma-Aldrich, T6199) followed by staining with FITC-conjugated secondary antibody (Jackson ImmunoResearch laboratories Inc., West Grove, PA). Nuclei were counterstained with DAPI. 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.

In vivo Tumor Model

Four to 6-week-old female nude mice (Fredrick Labs, Frederick, MD) 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 *ad libitum*. Tumor cells (5×10^6 cells) were injected subcutaneously into the right hind leg.

When tumors grew to a mean volume of 172 mm³, the mice were randomized to vehicle alone, AZD6244 alone, AZD6244 plus 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 performed using a Pantak (Solon, OH) irradiator with animals restrained in a custom jig. To obtain a tumor growth curve, perpendicular diameter measurements of each tumor were measured every 3 days with a digital calipers, and volumes were calculated using a formula $(L \times W \times W)/2$. Tumors were followed until the group's tumors reached a mean size of 1500 mm³. 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 1500 mm³ minus the number of days for the mean of the control group to reach the same size. Standard deviations (SDs) 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.

Western Blotting

Cell extracts were prepared using RIPA buffer (Pierce, Rockford, IL) containing protease inhibitors (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO), followed by measurement of protein concentrations by the Bradford method (Bio-Rad, Hercules, CA). 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 ERK 1/2 normalized to actin, the loading control in western blots of three cell lines.

Statistical Analysis

In vitro experiments were repeated three times and statistical analysis was done using a student's t-test. Data are presented as mean $\pm$ SD. A probability level of $P < 0.05$ was considered significant.

RESULTS

To determine the effects of AZD6244 on tumor cell radiosensitivity, clonogenic survival analysis was performed in the A549, MiaPaCa2, and DU145 cell lines. The AZD6244 concentration selected for each cell line was based on toxicity studies such that the dose resulted in approximately 50% toxicity as a single agent (100 nM for MiaPaCa2, and 250 nM for A549 and DU145). As shown in figure 1, AZD6244 treatment delivered 16 hours prior to IR increased A549, DU145, and MiaPaCa2 radiosensitivity with a dose enhancement factor (DEF) 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 two hours after irradiation. In conditions used for clonogenic assays, AZD6244 decreased radiation induced ERK1/2 phosphorylation in the A549, MiaPaCa2, and DU145 cell lines (figure 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 (γ H2AX), which has been established as a sensitive indicator of DNA DSBs with the resolution of foci corresponding to DSB repair (17). Cells were exposed to AZD6244 for 16 hrs and irradiated (4 Gy) as in the cell survival experiments, and γ H2AX foci were determined at 1, 6 and 24 hrs post IR. Exposure of cells to AZD6244 only for 16 hrs resulted in no significant increase in the number of γ H2AX foci in both the A549 and MiaPaCa2 cell lines (Supplemental figure S1). Irradiation (4 Gy) only induced a significant increase in the number of γ H2AX foci at 1 hr, which progressively declined to 24 hrs. Exposure to AZD6244 followed by 4 Gy resulted in a number of γ H2AX foci not significantly different to that observed with RT alone

at 1 hr thus AZD6244 does not impact the immediate DNA damage after irradiation. At 24 hrs the number of γ H2AX foci per cell was similar in the irradiation and combination group, thus AZD6244 does not inhibit DNA DSB repair.

Cell cycle analysis after pre-treatment with AZD6244 revealed no evidence of redistribution into radiosensitive phases of the cell cycle (figure 3A). Treatment with AZD6244 resulted in a lower percentage of cells in the G2/M phase of the cell cycle compared to 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 figure 3B, irradiation (4Gy) resulted in a rapid reduction in the mitotic index reaching a maximum decrease at 3 hrs 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 hrs 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 to that observed in vehicle treated cells (figure 4). In addition, treatment with AZD6244 reduced the expression of total Chk1 protein in unirradiated cells compared to that in vehicle treated unirradiated cells.

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 hrs after irradiation (4 Gy). As shown in figure 5A and B, there was a non-significant increase in apoptosis with both radiation and treatment with AZD6244 compared to untreated controls; however, the degree of apoptosis that was measured when combining AZD6244 and RT was less than additive in both the A549 and MiaPaCa2 cell lines. Thus the combination of AZD6244 and RT shown to enhance radiation-induced death in Figure 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 IR (4 Gy) and AZD6244 (as described in Methods and Materials) as well as the combination. As shown in figure 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 hrs. In cells receiving the combination treatment, a significant increase in the percentage of cells undergoing mitotic catastrophe were detected at 72 hrs post-treatment in both the A549 and MiaPaCa2 cell lines. This finding was accompanied by an increase in the proportion of cells containing greater than 4n DNA content by flow cytometry (Supplemental figure S2). An increase in cells containing more than 4n DNA was detected within 24 hours after radiation in both cell lines treated with vehicle or AZD6244. In addition, cells containing over 4n DNA

were significantly increased in A549 and MiaPaCa2 cells treated with AZD6244 compared to 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 subcutaneously (sc) 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); IR (3 Gy) only; and AZD6244 (50 mg/kg) administered by oral gavage 4 hrs before IR (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 figure 6A and B respectively. For each group, the time to grow from 172 mm³ (volume at the time of treatment) to 1500 mm³ was calculated using the tumor volumes from the individual mice in each group (mean $\pm$ SE).

For the A549 xenograft model, the time required for tumors to grow from 172 to 1500 mm³ 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 1500 mm³ to 35.6 ± 1.5 days. However, in mice that received the AZD6244 + IR combination the time for tumors to grow to 1500 mm³ 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 1500 mm³ 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 + IR 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 accounts 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 mm³ in mice exposed to the combined modality minus the time in days for tumors to grow from 172 to 1,500 mm³ in mice treated with AZD6244 only. The dose enhancement factor, obtained by dividing the normalized tumor growth delay in mice treated with AZD6244 + IR by the absolute growth delay in mice treated with radiation only, was 3.38 for 50 mg/kg of AZD6244.

A similar experiment was performed in MiaPaCa2 xenografts. The growth rates for the MiaPaCa2 tumors exposed to each treatment are shown in figure 6B. For the MiaPaCa2 xenograft model, the time required for tumors to grow from 172 to 1500 mm³ 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 1500 mm³ to 41.8 ± 2.3 days. However, in mice that received the AZD6244 + IR combination the time for tumors to grow to 1500 mm³ 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 1500 mm³ 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 + IR 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 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 MEK 1/2, provides a means to test this hypothesis with a clinically relevant molecule (10).

The data presented here indicate that AZD6244 enhances the radiosensitivity of a 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.

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

Since 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. Pre-treatment 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, post-irradiation cell cycle analysis revealed that treatment of cells with AZD6244 resulted in an increase in the mitotic index compared to 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 prior to irradiation led to a reduction in phosphorylated Chk1, likely a contributor to the abrogated G2 checkpoint.

Prolonged G2 arrest after genotoxic stress allows DNA damage repair prior to progression through mitosis (29,30). Although we observed an early increase in the mitotic index in AZD6244 treated cells compared to controls, we did not observe significant differences in the number of γ H2AX foci after 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 hr after irradiation with a similar mitotic index to vehicle treated cells at 24 hrs. Many cells treated with irradiation and AZD6244 or vehicle control had elevated γ H2AX foci at 1 and 6 hrs compared to 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 to 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.

Statement of Translational Relevance

This work reports the use of a clinically relevant molecule, AZD6244, as a radiation modifier. This agent inhibits MEK1/2 and has been successfully tested in Phase I and Phase II trials in patients with advanced cancer and is continuing to be tested in additional Phase II trials. This agent may be used as a radiation modifier in clinical trials in patients with tumors known to have activation of the Ras-Raf-MEK-ERK pathway via activating Ras mutations or EGFR pathway activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The effects of AZD6244 on tumor cell radiosensitivity

Cell lines A549 (A), MiaPaCa2 (B), and DU145 (C) were exposed to AZD6244 (250 nM for A549 and DU145, 100 nM for MiaPaCa2) or vehicle control for 16 hours, and irradiated with graded doses of X-rays. Colony-forming efficiency was determined 10 to 14 days later and survival curves generated after normalizing for cell killing by AZD6244 alone. The data represent the mean of three independent experiments. PE, plating efficiency with AZD6244; DEF, dose enhancement factor. Points, mean; bars, $\pm$ SE.

Figure 2. The effects of AZD6244 on ERK1/2 phosphorylation

The effects of AZD6244 on ERK1/2 phosphorylation. The A549 (A), MiaPaCa2 (B), and DU145 (C) cell lines were exposed to AZD6244 (250 nM for A549 and DU145, 100 nM for MiaPaCa2) or vehicle control for 16 hours, irradiated, harvested, and subjected to immunoblotting and densitometry. ERK1/2 phosphorylation was induced by radiation and reduced following exposure to AZD6244. C, control untreated cells; AZD, cells exposed to AZD6244 for 16 hours prior to harvesting or IR; 1h, 2h, 6h, time after exposure to IR. Densitometry was done using ImageQuant 5.2 software.

Figure 3. The effects of AZD 6244 on the cell cycle

A, A549 cells were exposed to 250 nM AZD6244 or vehicle control for 16 hours prior to evaluation of cell cycle distribution with flow cytometry. *, $P < 0.01$ according to student's t test (vehicle treatment vs. AZD6244 treatment). B, A549 cells were stained with propidium iodide at the specified times and analyzed using flow cytometry. To evaluate the activation of G2 cell cycle checkpoint, mitotic cells were distinguished from G2 cells, and the mitotic index was determined according to the expression of phosphorylated H3 detected in the 4N DNA content population by the flow cytometry. Loss of mitotic cells reflects the onset of G2 arrest. AZD6244, cells that had received AZD6244 (250 nM) alone. Columns, mean; bars, SE.

A

B

Figure 4. The effects of AZD6244 on Chk1 phosphorylation

A, A549 cells were exposed to AZD6244 (250 nM) or vehicle control for 16 hours, irradiated, and harvested, and subjected to immunoblotting. Chk1 phosphorylation was induced by radiation and reduced following exposure to AZD6244. C, control untreated cells; AZD, cells exposed to AZD6244 for 16 hours prior to harvesting or IR; 1h, 2h, time after exposure to IR. B, Densitometry was done using by ImageQuant 5.2 software.

Figure 5. The effects of AZD6244 on the mechanism of cell death after irradiation

Apoptosis: Cells were treated with vehicle control or AZD6244 (250 nM) and harvested at the specified times. Treated cell samples were added to a 150 μ L staining solution (Guava Nexin Assay) containing 135 μ L 1x apoptosis buffer, 10 μ L Annexin V-PE, and 5 μ L of 7-AAD. Samples (2,000 cells per sample) were evaluated by flow cytometry, A549 (A), MiaPaCa2 (B). Columns, mean; bars, SE. **Mitotic Catastrophe:** Cells growing in chamber slides were exposed to AZD6244 (250 nM) or vehicle control, irradiated (4 Gy), and fixed at the specified times for immunocytochemical analysis for mitotic catastrophe, A549 (C), MiaPaCa2 (D). Nuclear fragmentation was evaluated in 150 cells per treatment per experiment. Nuclear fragmentation was defined as the presence of two or more distinct lobes within a single cell. Columns, mean; bars, SE. Nuclear fragmentation was defined as the presence of two or more distinct lobes within a single cell. *, $P < 0.01$ according to student's t test (IR vs. AZD6244 + IR).