Cancer Therapy: Preclinical

The Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase 1/2 Inhibitor AZD6244 (ARRY-142886) Enhances the Radiation Responsiveness of Lung and Colorectal Tumor Xenografts

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Abstract

Purpose: Novel molecularly targeted agents, given in combination with radiotherapy, have the potential to increase tumor response rates and the survival of patients with lung cancer. AZD6244 is a potent and selective inhibitor of mitogen-activated protein kinase (MAPK) kinase 1/2 (MEK1/2), a critical enzyme within the MAPK/extracellular signal-regulated kinase (ERK) signaling pathway that regulates the proliferation and survival of tumor cells.

Experimental Design: This study examined the potential benefit of combining AZD6244 with fractionated radiotherapy using human lung and colon carcinoma xenograft models.

Results: AZD6244 reduced ERK phosphorylation in Calu-6 lung cancer cells in vitro. Administration of AZD6244 for 10 days (25 mg/kg twice daily p.o.) inhibited the tumorigrowth of Calu-6 xenografts, with regrowth occurring on cessation of drug treatment. When fractionated tumor-localized radiotherapy (5 x 2 Gy) was combined with AZD6244 treatment, the tumor growth delay was enhanced significantly when compared with either modality alone, and this effect was also seen in a colon tumor model. We examined the effect of inhibiting MEK1/2 on the molecular responses to hypoxia, a potential interaction that could contribute to radioresponsiveness. AZD6244 reduced hypoxia-inducible factor-specific transactivation in vivo, shown using Calu-6 dual clone cells that stably express a Firefly luciferase gene under the control of a hypoxia-driven promoter. Furthermore, hypoxia-inducible factor-1α, GLUT-1, and vascular endothelial growth factor levels were reduced by AZD6244, and there was a significant decrease in vascular perfusion in the tumors given combination treatment when compared with the other treatment groups.

Conclusions: These data provide support for the clinical development of AZD6244 in combination with radiotherapy and indicate a potential role for AZD6244 in inhibiting the tumor hypoxia response. (Clin Cancer Res 2009;15(21):6619-29)

Ionizing radiation (IR) is used as a primary treatment for many types of cancer, including those of the lung, breast, and prostate. Among the factors important for tumor radiation responsiveness are the level of tumor hypoxia and the intrinsic radiosensitivity of the tumor cells (1, 2). As approximately half of all patients with cancer undergo radiotherapy as part of their treatment (3), enhancing therapeutic outcome for even a small percentage of these patients has the potential to translate into significant clinical effect. In addition to radiotherapy, many novel anticancer agents are being developed with the aim of targeting proteins such as kinases, the activities of which are deregulated within tumor cells (4–6). The potential to combine such inhibitors of aberrant signaling pathways with localized IR is considerable, although it is necessary to evaluate whether such approaches could act cooperatively with radiotherapy and to examine any potential scheduling interactions to optimize therapeutic outcome (7).

One of the signaling pathways considered to have a key role in cancer progression is the RAS/RAF/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (or MAPK/ERK) pathway. MAPK signaling through the ERK1/2 proteins is known to control several aspects of cellular physiology, including DNA synthesis, cell cycle entry, cell proliferation, differentiation, and apoptosis (8, 9). In tumor cells, this pathway

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Translational Relevance

Approximately half of all patients with cancer receive radiotherapy during the treatment of their disease. The identification of novel therapeutic approaches that can be combined with and are complementary to radiotherapy may therefore afford the potential to improve significantly on current clinical outcomes. This preclinical study is the first to report significant antitumor benefits from combining daily oral dosing of AZD6244, a potent and selective inhibitor of mitogen-activated protein/extracellular signal-regulated kinase kinase 1/2, with a clinically relevant fractionated radiotherapy protocol in human tumor xenograft models. An examination of the molecular mechanisms involved indicates that modulation of the hypoxia signaling response is likely to contribute toward the observed therapeutic benefits. These data suggest that clinical examination of mitogen-activated protein/extracellular signal-regulated kinase 1/2 inhibition with radiotherapy is warranted.

can be activated constitutively by cell surface growth factor receptors or downstream activating mutations in Ras and B-RAF (6). Approximately one third of human cancers have mutations in Ras, primarily the K-RAS isoform (10, 11). MAPK signaling might also potentially influence tumor cell radiosensitivity because the activity of ERK1/2 is associated with the radiation-induced DNA damage response (12). Ras mutations can confer a radiation-refractory phenotype (13, 14), and MAPK signaling can be stimulated by treatment with IR in some tumors (15–18). Radiation-induced MAPK pathway activation has also been shown to regulate the production of vascular endothelial growth factor-A (VEGF-A; ref. 19), which promotes tumor angiogenesis and neovascular survival (20), and is implicated in endothelial radiosensitization; considered by some to be one of the primary determinants of tumor radiosensitivity (21, 22). The VEGF promoter region contains a hypoxia response element (HRE), and one of the principal regulators of VEGF-A production is hypoxia-inducible factor-1α (HIF-1α; refs. 23, 24), which is also a target of ERK1/2. The ERK proteins regulate HIF-1α protein synthesis (25) and transcriptional activity by phosphorylating sites on the HIF-1 transcriptional activation domain (26–28) and on its coactivator p300/cyclic AMP–responsive element-binding protein (29). Furthermore, HIF-1 activity can promote tumor radioresistance through a variety of mechanisms (30, 31).

AZD6244 (ARRY-142886) is a selective, novel, and highly potent small molecule inhibitor of MEK1/2 (5) that has oral bioavailability and is currently in phase II trials. Yeh et al. (5) showed that AZD6244 functions by binding to the allosteric inhibitor-binding site on MEK, and is highly specific with an IC50 against MEK1 of 14.1 nmol/L with no observed inhibition at 10 μmol/L against more than 40 other serine/threonine kinases. A recent study reported no significant difference between AZD6244 and pemetrexed for the second-line and third-line treatment in patients with advanced non–small cell lung carcinoma (32). The purpose of this study was to examine the effect of combining AZD6244 with fractionated radiotherapy in human tumor models (Calu-6 lung and HCT116 colon tumor xenografts). The combination was found to impart significantly greater antitumor activity than either therapy alone in vivo, an effect that may be manifested both by the ability of AZD6244 to directly sensitize tumor cells to the cytotoxic effect of radiation and to modulate tumor vessel functionality.

Materials and Methods

Cell culture. Calu-6 human non–small cell lung carcinoma and HCT116 colon carcinoma cells were obtained from the American Type Culture Collection. Calu-6 cells were also stably transfected with a dual luciferase cassette containing two reporter constructs to form Calu-6 dual clone (DC) cells; a Firefly luciferase gene under the control of a HRE derived from LDH-A as reported previously (33) and a Renilla luciferase gene under the control of a constitutive promoter (EF-1) as an internal control. Cell culture reagents were obtained from Life Technologies (Invitrogen, Ltd.) except for FCS (Biosera). Cells were plated in 7T5 tissue culture flasks, grown as monolayer cultures, and maintained in RPMI 1640 supplemented with 10% FCS and 2 mmol/L of glutamine (no antibiotics were used in routine cell cultures). Cells were routinely screened for the presence of Mycoplasma using a commercial assay (MycoTest Life Technologies, Invitrogen, Ltd.)

Treatment conditions. For in vitro treatments, cells were seeded in the appropriate plates and allowed to adhere overnight in standard culture conditions (95% air and 5% CO2 at 37°C). AZD6244 [ARRY-142886 (Array Biopharma) AstraZeneca; 10 mmol/L stock in DMSO] was serially diluted in DMSO, diluted into culture medium, and then added to cells. After a specified time period, plates were maintained in standard culture conditions or placed in sealed aluminum chambers with a single inlet and outlet valve and continually gassed with 1% O2 in a N2 plus 5% CO2 mix for the designated time. For radiation treatments, plates were transferred to a radiation source (Faxitron X-ray). A 2, 4, or 6 Gy X-ray dose was given at a rate of 1 Gy/min under ambient conditions in air.

Western blotting. Cells were seeded at 1 × 105 cells in 10-cm dishes and allowed to adhere overnight. AZD6244 was added to the cells at the indicated concentration and the plates were returned to standard culture conditions for 30 min before exposure to hypoxic conditions or irradiation as described previously. Lysates from cells and tumors were prepared and protein concentration was determined as described previously (31). Snap-frozen tissues in liquid nitrogen were lysed using lysis buffer (10 mmol/L HEPES, 1.5 mmol/L MgCl2, 10 mmol/L KCl, 0.05 mmol/L DTT; Sigma), briefly homogenized, and the lysates cleared by centrifugation at 10,000 × g for 15 min at 4°C. Equal amounts of protein (30–50 μg) were separated by 10% SDS-PAGE and Western blotting was done as described previously (31). Membranes were probed with the following antibodies: pERK (Thr202/Tyr204) and total ERK antibodies (1:500; Cell Signaling Technology), cleaved poly(ADP) ribose polymerase (PARP; 1:1,000; Cell Signaling Technology), HIF-1α (1:500; BD Biosciences), GLUT-1 (1:3,000; Millipore), and β-actin (1:100,000; Sigma).

Clonogenic assays. Calu-6 DC cells were seeded at 1 × 105 in six-well plates and allowed to adhere overnight. In the simultaneous schedule, AZD6244 was added to cells and the plates irradiated immediately before returning to standard culture conditions for 48 h before plating at low seeding densities in six-well plates. In the drug pretreatment schedule, AZD6244 was added to cells for 48 h before irradiation and plated immediately. Appropriate controls were done for each schedule. The colonies were stained with methylene blue 10 to 12 d later and the colony-forming efficiency following treatment was related to that of the relevant controls.

Dual luciferase assay. Calu-6 DC cells were seeded at 5 × 104 in 96-well plates and allowed to adhere overnight. AZD6244 was added to
cells for 4 h under standard culture conditions before being left in aerobic conditions or placed in hypoxic conditions for 18 h. Cells were lysed with 1× passive lysis buffer (Promega) and HRE-driven Firefly and EF1-driven Renilla luciferase activity analyzed simultaneously by the Dual Luciferase Reporter assay system (Promega) using the manufacturer’s approved reagents and protocols. Tumor lysates obtained as described previously were used in the dual luciferase assay as above, and the luminescent signal obtained from the expression of Firefly was divided by that obtained for the Renilla to normalize for the effects on general (constitutive) transcription. The Firefly/Renilla ratios in the tumor lysates were normalized for protein concentration.

VEGF ELISA. Supernatants from Western immunoblotting (24 h) samples were collected and stored at -20°C and lysates from tumor xenografts were obtained as described previously. Protein concentration was determined and human VEGF levels in cell culture supernatants and tumor lysates were determined by ELISA using a Quantikine Human VEGF Immunoassay (R&D Systems) according to the manufacturer’s recommended protocols.

Tumor models. Calu-6 DC and HCT116 cells were harvested in exponential phase growth. For tumor implants, Calu-6 cells were prepared at 5 × 10⁷ cells/mL in serum-free RPMI. A 0.1-mL volume of cell suspension was implanted intradermally on the back of female nu/nu CBA mice of age 10 to 12 wk to initiate tumor xenografts. Once a palpable tumor formed, measurements were taken daily using calipers and the tumor volume was determined as follows: tumor width × length × depth.

Dorsal window chamber. The procedure followed for the introduction of dorsal window chambers has been described previously (34). Drug and/or radiation treatments were initiated ~10 d after cell implantation when substantial vascularization was apparent within the growing tumor mass.

Drug and/or radiation treatment schedules. Mice bearing established tumors of 240 to 300 mm³ (n = 5 per group) were randomly assigned to designated treatment groups. The sample size per group was derived using archived data and was sufficient to detect a 50% change in treatment response with 80% power to a significance level of P = 0.05. AZD6244, solubilized in a methocel/polysorbate buffer or vehicle (buffer alone), was administered twice daily (8 h apart, morning and evening) by oral gavage (0.1 mL/10 g body weight) at a dose of 25 mg/kg. Tumor-localized radiotherapy (X-ray) was administered as five daily 2-Gy fractions under ambient conditions to nonanesthetized mice restrained in lead shielded containers as described previously (34). Radiation was given 2 h after the first daily dose of AZD6244 or vehicle. Tumor volumes and weights were measured daily until the tumor volume reached 1,000 mm³, which was designated as the experimental endpoint for ethical reasons. The data calculated from the growth profile of each individual tumor included the number of days the tumor took to triple in volume from the original tumor volume at the start of treatment (relative tumor volume × 3; RTV3) and the growth delay in achieving RTV3 (time to RTV3 in treated tumors minus time to RTV3 in vehicle-treated control tumors).

To assess the effect of treatment on hypoxia response, tumors were harvested after 5 d of twice daily treatment with vehicle or AZD6244 (25 mg/kg; days 0-4), and to assess the effect on vascular perfusion, tumors were harvested after 10 d of AZD6244 (days 0-9) and/or radiation (5 × 2 Gy; days 0-9), and controls were harvested at treatment size. All procedures used in this study incorporated the 1998 United Kingdom Coordinating Committee on Cancer Research Guidelines and were in compliance with The Scientific Procedures Act of 1986. Studies were approved by the Home Office Inspectorate and the University of Manchester Ethics Care Committee under PPL 40/2328. Animals were maintained using the highest possible standard of care and priority was given to their welfare above experimental demands at all times.

Administration of perfusion and hypoxic markers. To assess perfusion, Hoechst 33342 (0.1 mL of a 6 mg/kg stock; Sigma) was administered via i.v. (tail vein) injection to mice with a tumor volume of 250 to 300 mm³ for controls or following 10 d of treatment with AZD6244 and/or radiotherapy (n = 5 per group) 1 min before excision. The hypoxia marker pimonidazole (0.2 mL of a 10 mg/mL stock; i.p.; Chemicon International, Inc.) was administered to the same cohorts of mice 2 h before tumor excision. Tumors were excised rapidly and snap-frozen in liquid nitrogen. For window chamber studies, mice were given bovine serum albumin (BSA) tagged with Alexa fluorochrome (BSA-Alexa-Fluor; Molecular Probes, Invitrogen). Background fluorescence was monitored by intravital microscopy and time-lapsed images recorded following i.v.
injection of BSA-Alexa-Fluor (1 mg/mL in sterile saline; 0.1 mL/mouse) for 10 min.

**Analysis of perfusion, vasculature, and hypoxic fraction.** Cryostat sections (8 μm) were prepared from snap-frozen tumor material. Tumor tissue sections were first scanned using a fluorescent microscope to determine the number of Hoechst-stained (perfused) vessels. Sections were then fixed in ice-cold acetone and endothelial cells were stained using rat anti-CD31 (BD Biosciences) followed by TRITC-labeled goat anti-rat antibody (Molecular Probes, Invitrogen) as described previously (34). Hypoxia was detected in adjacent sections using Hypoxyprobe antibody (Chemicon International) followed by rabbit anti-mouse FITC (DAKO) as described previously (34). Tissue sections were analyzed using a NIKON Eclipse E800 with associated MetaMorph software. Vessel density was analyzed per unit area of the tumor section, and hypoxic fraction as a percentage of the tumor area exhibiting pimonidazole staining.

**Statistical analysis.** Statistical comparison between more than two groups was carried out by ANOVA followed by a least significant difference *post hoc* test, and between two groups by an independent sample *t* test using the SPSS statistical software package (SPSS, Inc.). No adjustments were made for multiple comparisons in the analyses. To analyze whether there was a significant interaction between treatments in the combination study, a two-way ANOVA test was carried out using SigmaStat. The data analyzed was log (final tumor volume) - log (initial tumor volume) calculated for each individual group on the final day of the study when data were available for all groups. Throughout the article, data presented are averages of three independent experiments and are expressed as mean ± SE and were significant at *P* < 0.05.

**Results**

AZD6244 inhibits ERK activity and increases cleaved PARP levels in Calu-6 cells alone and in combination with radiation. Phosphorylated ERK (pERK) status is a useful biomarker to confirm the inhibition of MEK1/2 activity. As a consequence of MEK1/2 inhibition, AZD6244 has previously been shown to have a proapoptotic effect in some preclinical tumor models including Calu-6 (35). First, we examined whether Calu-6 wild-type (WT) and DC cells were equally sensitive to AZD6244. Western immunoblotting showed high levels of pERK in untreated Calu-6 WT and DC cells, presumably due to mutant K-Ras–driven constitutive activation of the MAPK pathway (Fig. 1A). The expression of pERK was reduced in both cell types following

**Fig. 2.** Effects of AZD6244 alone or in combination with radiation on Calu-6 DC tumor xenografts. A, AZD6244 decreases pERK levels in the Calu-6 DC xenograft model. Mice bearing Calu-6 tumors were given one dose of vehicle or AZD6244 (25 mg/kg; p.o.) and tumors were harvested 4 h later. Lysates from the excised tumors were analyzed for pERK and total ERK expression by Western blotting. B, combination AZD6244 and radiotherapy enhances the therapeutic response of Calu-6 DC xenografts compared with either therapy alone, independently of whether fractionated radiotherapy is administered from the beginning or middle of the 10-d AZD6244 treatment. AZD6244 was given twice daily (25 mg/kg; p.o.) at 8-h intervals for 10 d (days 0–9; broken arrow). Tumor growth after the removal of AZD6244 mirrored that of vehicle-treated control tumors. Radiotherapy was given as 5 × 2 Gy fractions at 24-h intervals (days 0–4) in combination with vehicle or AZD6244; when used in combination, the 2-Gy radiation dose was given 2 h after the first daily dose of AZD6244. In the first combination group, radiation was given during the first 5 d of AZD6244 (days 0–4; black line), and in the second combination group during the second 5 d of AZD6244 (days 5–9; gray line). Points, average tumor values plotted until the time when the first tumor within the group reached 1,000 mm³; bars, SE. C, combination AZD6244 and radiotherapy increases the tumor growth delay compared with either therapy alone (*, *P < 0.05 versus AZD6244/ radiation alone). Tumor growth delay (RTV3treated - RTV3control) values are shown. A two-way ANOVA test on the last day when data were available for all groups (day 15) showed a significant interaction between AZD6244 and radiation treatment (*P = 0.035).
incubation with ≥2 nmol/L of AZD6244 for 3 hours, demonstrating that the compound could inhibit constitutive MEK1/2 activity. We then examined the effect of radiation alone or in combination with AZD6244 on Calu-6 DC cells. A concentration of 200 nmol/L of AZD6244 (for 24 hours) increased cleaved PARP levels while decreasing pERK levels (Fig. 1B), indicating that in this cell line, inhibition of MEK1/2 induces an apoptotic response. Radiation (4 Gy) did not affect pathway activation or drug-induced reduction of pERK (24 hours after IR and following continuous exposure to AZD6244), and did not significantly affect cleaved PARP levels induced by AZD6244. Time points of 5, 20, 40, 60, 120, and 240 minutes after irradiation were also examined without any apparent effect of IR on pERK levels (data not shown).

**Pretreatment with AZD6244 enhances the radiosensitivity of Calu-6 cells.** As AZD6244 induces the expression of apoptotic markers in Calu-6 cells, we next investigated the effect of scheduling AZD6244 treatment at the same time as or 48 hours before radiation using clonogenic assays in vitro. Hagan et al. (16) previously showed that the ability of MAPK inhibition to sensitize cells to clonogenic assays required a prolonged ≥48-hour inhibition of MAPK function. In both schedules, Calu-6 DC cells were treated for 48 hours with AZD6244, combined with irradiation (2, 4, or 6 Gy) and analyzed for their radiosensitivity. In Fig. 1C, data were normalized for the effects of drug treatment alone (survival fraction of 200 nmol/L AZD6244, 0.62 ± 0.11). The schedule involving 48 hours of AZD6244 treatment before irradiation increased tumor cell radiosensitivity, and no such effect was seen when the simultaneous schedule was used (Fig. 1C).

**Combining AZD6244 and radiotherapy in vivo increases the therapeutic benefit compared with either modality alone.** We next examined the efficacy of AZD6244 alone or in combination with radiotherapy in Calu-6 DC xenografts. Target inhibition was shown in tumor-bearing mice treated with one dose of vehicle or AZD6244 (25 mg/kg; p.o.). Tumors were harvested 2 hours later and snap-frozen. Immunoblotting of the tumor lysates showed AZD6244-induced inhibition of ERK phosphorylation compared with tumors from vehicle-treated control animals (Fig. 2A). The effect of AZD6244 (25 mg/kg twice daily) treatment on Calu-6 WT and DC tumor xenografts was then investigated, and the 5-day treatment with AZD6244 had a similar effect in both models (Supplementary Fig. S1). In the combination therapy study, vehicle-treated Calu-6 DC xenografts grew rapidly (Fig. 2B). Twice daily dosing with AZD6244 (25 mg/kg) for 10 days (days 0-9) inhibited tumor growth, with a significant increase in time to RTV3 (27.5 ± 3.1 days) compared with controls (13.6 ± 2.1 days). The nadir tumor volume (i.e., smallest volume following start of treatment) of drug-treated mice was 117.5 mm³ on average, occurring 24 hours after treatment ended. However, 48 hours after drug withdrawal, the tumors began to regrow at a rate similar to controls, and reached the original treatment tumor volume ~7 days after treatment had ended. Fractionated radiotherapy induced a typical radiation growth response, with a delayed but prolonged effect on tumor growth (RTV3, 24.3 ± 2.6 days). Radiation-treated tumors (5 × 2 Gy; days 0-4) regressed to 290 mm³ from a maximal volume of 345 mm³ 6 days after radiation ended; larger than the average original treatment tumor volume.

Two combination treatment schedules were used; fractionated radiotherapy administered during the first (days 0-4) or last 5 days (days 5-9) of the 10-day AZD6244 treatment. It was hypothesized that prolonged (5 days) MEK inhibition before the introduction of radiotherapy might enhance radiosensitivity, as suggested by the in vitro clonogenic assay data. Both combination schedules gave a more pronounced regression than drug alone; the tumor volume was maintained at ~50 mm³ for 9 days after treatment ended in the group receiving drug and IR from the same start date, and did not reach the original tumor treatment volume until 20 days after treatment ended (Fig. 2B). On day 15 (6 days after treatment ended), there was a statistically significant interaction between radiation and AZD6244 treatment (P = 0.035). The two combination schedules had very similar effects with a time to RTV3 of ~43 days for both groups, and the tumor growth delay was significantly greater than that achieved using AZD6244 or radiation treatment given independently (P < 0.05; Fig. 2C). Combination therapy was well tolerated; the maximum body-weight loss was 3.34% for IR during the first 5 days of AZD6244, and 3.55% for IR during the last 5 days of AZD6244.

The experiment was repeated using a HCT116 colon cancer xenograft model, with radiation given during the first 5 days of AZD6244 in the combination group. AZD6244 alone was less effective than in the Calu-6 model (Fig. 3A) and did not cause regression during the course of treatment. However, when AZD6244 was combined with radiotherapy, regression was
observed during the treatment period. The nadir volume observed was 178 ± 29 mm³ and this occurred 24 hours after the end of drug treatment. Average tumor volumes at the start of treatment were 248 ± 8 mm³. As with the Calu-6 model, the tumor growth delay observed in the combined treatment group was again significantly greater than that achieved using AZD6244 or radiation independently (P < 0.05; Fig. 3B).

**AZD6244 reduces HIF-specific transactivation and HIF-1α, GLUT-1, and VEGF protein levels.** The MAPK pathway may affect HIF-1 functionality (26–29), which has the potential to affect tumor radioresponsiveness (30, 31). As AZD6244 and radiotherapy had a marked effect in the Calu-6 xenograft model, the effect of MEK1/2 inhibition on hypoxia response using AZD6244 was examined. Drug-induced changes in HIF-specific transactivation under hypoxic (1% O₂) conditions were assessed in Calu-6 DC cells using a HRE-luciferase reporter assay, which measured the activity of *Firefly* and *Renilla* (EF-1-driven) luciferase activities in a dual-luciferase assay. A representative graph from a single experiment that was repeated four times is shown. B, levels of pERK, total ERK, HIF-1α, and GLUT-1 were determined by Western blotting of the cell lysates. C, VEGF levels in the supernatants were measured by ELISA.

**Fig. 4.** AZD6244 reduces the in vitro hypoxia response. Cells were treated with vehicle control or concentrations of AZD6244 as indicated 30 min before exposure to hypoxic (1% O₂) conditions for 24 h. Cells were maintained in vehicle/drug-containing medium throughout. The supernatants were collected and the cells were lysed. A, cell lysates were analyzed for *Firefly* (HIF/HRE-driven) and *Renilla* (EF-1-driven) luciferase activities in a dual-luciferase assay. A representative graph from a single experiment that was repeated four times is shown. B, levels of pERK, total ERK, HIF-1α, and GLUT-1 were determined by Western blotting of the cell lysates. C, VEGF levels in the supernatants were measured by ELISA.
AZD6244 under hypoxic conditions. To assess the effect of AZD6244 on HIF-1α protein levels and its downstream target GLUT-1, cells were incubated under aerobic or hypoxic (1% O₂) conditions for 24 hours in the presence or absence of AZD6244. HIF-1α and GLUT-1 protein levels were increased in untreated cells under hypoxic conditions compared with aerobic controls (Fig. 4B). AZD6244 concentrations of ≥20 nmol/L reduced levels of HIF-1α under hypoxic conditions, and concentrations of ≥200 nmol/L reduced GLUT-1 levels.

Signaling through HIF-1 (23, 24) and ERK (36) could increase VEGF expression, and Park et al. (19) previously showed that radiation-induced MAPK signaling could enhance the levels of VEGF production. Inhibitors of VEGF signaling have been shown to increase the efficacy of radiotherapy in tumor xenografts (34, 37, 38) and in clinical studies (39). Based on these data, and our results showing that AZD6244 reduces the HIF response, we assessed the effect of AZD6244 on VEGF levels as a contributory mechanism to the enhanced therapeutic benefits observed when the drug was used in combination with radiotherapy.

**Figure 5.** AZD6244 reduces the in vivo hypoxia response. Mice bearing Calu-6 DC tumors were dosed with vehicle or AZD6244 (25 mg/kg twice daily; p.o.) for 5 d; the tumors were harvested and lysed 4 h after the first dose of AZD6244 on the 5th day of treatment. Data shown represent the average Firefly/Renilla values for tumor lysates from three control or AZD6244-treated mice. A, dual luciferase assay determined the ratio of Firefly to Renilla activity in the tumor lysates. B, levels of pERK, total ERK, HIF-1α, and GLUT-1 were determined by Western blotting of the tumor lysates. C, VEGF levels in the tumor lysates were measured by ELISA.
radiation. Secreted VEGF levels were increased 2.6-fold in the supernatant of cells exposed to hypoxia for 24 hours compared with aerobic cells, and 20 nmol/L of AZD6244 significantly decreased the levels of VEGF produced under hypoxic conditions ($P < 0.05$; Fig. 4C).

As AZD6244 was shown to affect the in vitro hypoxia response, its effect was then assessed in vivo using tumors from mice treated for 5 days with twice daily vehicle or AZD6244 (25 mg/kg). AZD6244 inhibited HIF-specific transactivation as shown by significantly lower Firefly values in tumors from mice treated with AZD6244 compared with vehicle-treated controls ($P = 0.02$), whereas Renilla values were not significantly affected ($P = 0.11$; Fig. 5A). Furthermore, there was a decrease in the levels of both HIF-1$\alpha$ and GLUT-1 in the AZD6244-treated tumors (Fig. 5B), in conjunction with a decrease in pERK expression. The human VEGF content from the tumor lysates was then measured, demonstrating a significant reduction in tumors from mice treated with AZD6244 for 5 days compared with vehicle-treated controls (Fig. 5C).

AZD6244 and radiation combination therapy decreases vascular perfusion in vivo. Radiation doses as low as 2 Gy could inhibit endothelial cell proliferation, survival, and invasion in vitro (40). As we showed that AZD6244 reduces the levels of VEGF that, when present, can protect from the damaging effects of radiation (21, 22), the effect of combination therapy on vascular density and perfusion was assessed. A histology...
study was carried out using mice treated for 10 days with AZD6244 (25 mg/kg twice daily; days 0-9), fractionated radiotherapy (5 × 2 Gy), and combination therapy with radiation given during the first 5 days with AZD6244 (days 0-4). All tumors were harvested on day 9, except for controls which were harvested at treatment size. When measurements were made at this stage, there was no significant difference in total vessel density between any of the groups (P > 0.05; Fig. 6A). However, there was a significant decrease in vascular perfusion in the radiation-treated tumors compared with the other groups (P < 0.05), as assessed by counting the number of Hoechst-33342-stained (perfused) vessels (Fig. 6B). A combination of AZD6244 and radiotherapy caused a further significant decrease in vascular perfusion compared with radiotherapy alone (P < 0.05; Fig. 6A).

The extent of tumor hypoxia, evidenced by immunohistochemical analysis of pimonidazole binding, was analyzed in the same tumors (Fig. 6B). Although AZD6244 alone had little effect on vessel density and perfused fraction, a significant decrease in hypoxic fraction was revealed (Supplementary Fig. S2; Fig. 6B). The decrease was further exacerbated in the combined treatment group, in which the level of hypoxia observed was significantly lower than both control (P = 0.002) and radiation-treated tumors (P = 0.02). Radiation alone elicited a small but insignificant reduction in the extent of tumor hypoxia (Fig. 6B).

To make a direct real-time assessment of the effects of AZD6244 and radiotherapy on tumor vessels compared with treatment with either modality alone, Calu-6 DC xenografts were grown in dorsal window chambers. Once tumors had been established, mice were given AZD6244 (25 mg/kg) twice daily for 6 days (days 0-5) with or without radiotherapy (3 × 2 Gy) during the first 3 days of AZD6244 treatment (days 0-2). Radiation was also given alone according to the same fractionated regimen. Before the first drug treatment (day 0) and on the third and final day of treatment (days 2 and 5), BSA-Alexa-Fluor was administered by tail vein injection and tumor uptake was examined by intravital fluorescent microscopy (Fig. 6C and D). The average change in fluorescent uptake on day 0 was 2.5 ± 0.5-fold (range, 1.26-4.26) over the background. The uptake at days 2 and 5 of treatment was then compared with that observed at day 0 (Fig. 6C) for each individual treated tumor. Consistent with the histologic studies, real-time analysis of vascular function revealed that combined treatment with radiotherapy and AZD6244 led to substantial reductions in BSA-Alexa-Fluor uptake compared with tumors treated with radiation or AZD6244 alone. Three fractions of radiation (Fig. 6C) did not have as noticeable an effect on vascular perfusion as 5 × 2 Gy (Fig. 6A). Regardless of this, however, three fractions were sufficient to inhibit perfusion when combined with AZD6244 (Fig. 6D).

Discussion

The identification of activating RAS and B-RAF mutations and elevated levels of ERK1/2 phosphorylation in tumors show the significant role that the MAPK signaling pathway plays in human cancers (6, 10, 41). In this study, the effect of combining radiation and AZD6244 was investigated in vivo and in vitro. AZD6244 is a potent and orally active inhibitor of MEK1/2 activity (5), which blocks ERK phosphorylation and increases the expression of apoptotic markers in the KRAS mutant Calu-6 DC cell line (Fig. 1). Davies et al. (35) recently showed a similar effect in the Calu-6 WT model. AZD6244 specifically inhibits MEK1/2 as shown recently by Yeh et al. (5), and has no significant effect on the MEK5/ERK5, mammalian target of rapamycin, p38, or c-Jun-NH2-kinase signaling pathways (42). AZD6244 induced tumor regression in vivo (Fig. 2B), and immunoblotting confirmed the inhibition of pERK in response to the drug (Fig. 5B). In the present study, tumors in AZD6244-treated mice began to regrow upon cessation of drug treatment (Fig. 2B), which led us to examine whether there was additional benefit from combining the drug with radiation. Current clinical trial results suggest that monotherapy with AZD6244 seems to be insufficient to provide optimal antitumor therapy in non–small cell lung carcinomas (32), and a recent preclinical study suggested that AZD6244 may have improved efficacy when used in combination with conventional anticancer therapies (43).

As AZD6244 targets proliferating tumor cells with a constitutively active MAPK pathway, the hypothesis exists that administration of the drug during a fractionated radiation regimen may further reduce the number of surviving clonogenic cells. These cells have the potential to repopulate the tumor between radiation fractions; one of the main causes of radioresistance and local tumor control failure. Radiation exposure has been shown to activate the MAPK pathway in some cell lines (15–18). The reason we did not observe an IR-induced increase in ERK activity at the protein level at least (Fig. 1B) may be due to a dominant, constitutive hyperactivation of the pathway in this cell line due to mutant KRAS coupling to RAF. Our clonogenic assay results show that pretreatment with AZD6244 increased tumor cell radiosensitivity in vitro (Fig. 1C). Hamed et al. (44) showed that 48-hour pretreatment of mammary tumor cells with the MEK inhibitor PD184352 and an anticancer staurosporine analogue UCN-01 followed by 24-hour drug removal before irradiation enhanced cell death compared with either drug or IR alone. Furthermore, very recent studies showed that AZD6244 given 16 hours before radiation treatment enhanced the radiosensitivity of lung (A549), pancreatic (MiaPaCa), and prostate (DU145) cells in vitro (45). The mechanistic basis for this sensitization does not seem to involve enhanced apoptosis, with no change in PARP cleavage observed between cells treated with IR alone or in combination with AZD6244.

When AZD6244 was combined with fractionated radiotherapy in vivo, a significant increase in tumor growth delay was observed compared with either monotherapy, regardless of whether radiation was given during the first 5 or last 5 days of the 10-day AZD6244 treatment (Fig. 2B). This shows that extended MEK inhibition before radiotherapy is unnecessary, which differs from our in vitro results and the work of Hamed et al. (44), who showed that irradiation of PD184352 and UCN-01–treated tumors 24 hours after the cessation of drug treatment significantly enhanced the therapeutic outcome compared with concomitant therapy. Radiation was administered 2 hours after AZD6244 in our experiments, at which time ERK was inhibited (Fig. 2A) and remained so during the course of treatment (twice daily dosing for 5 days; Fig. 5B). Chung et al. (45) also observed an enhanced radiotherapeutic response in vivo when a single dose of AZD6244 (50 mg/kg) was administered 4 hours before a single dose of radiotherapy (3 Gy).
It is therefore probable that the inhibition of ERK is essential during radiotherapy in vivo, and that AZD6244 decreases radioresistance to some extent by targeting cells with clonogenic potential.

We observed an extended period of regrowth in the tumors treated with combination AZD6244 and radiation, which had a tumor growth delay of ~29 days compared with 14 days for AZD6244 and 11 days for radiation (Fig. 2C). Markers for DNA damage (phosphorylated H2AX) and apoptosis (cleaved caspase-3) were assessed by immunohistochemistry after 5 days of treatment, but did not reveal any significant difference between the combination and other groups (data not shown). This is consistent with other recent data, which also suggested that the interaction between AZD6244 and radiotherapy was independent of the effects on DNA double-strand break repair (from examining phosphorylated H2AX) or apoptosis (quantified with an Annexin V binding assay), but may correlate with an increase in mitotic catastrophe (45). However, unlike the Ras mutant cell lines in the study of Chung et al. (45), AZD6244 alone or in combination with radiotherapy had no effect on the cell cycle profile of Calu-6 cells (data not shown).

We also examined a HIF-related mechanism as the MAPK/ERK signaling pathway regulates the activity of HIF-1 (26–29) and VEGF (19, 36), both of which confer radioresistance to tumor and endothelial cells (21, 22, 31, 37, 46). We showed an AZD6244-induced reduction in HIF-1α expression and functionality in vivo and in vitro, as well as a reduction in the HIF downstream targets GLUT-1 and VEGF (Figs. 4 and 5). The in vitro results differ with those of Sutton et al. (47), who recently suggested that although ERK1/2 signaling is important for HIF-1α induction and activity in response to insulin-like growth factor-1; it is not essential for these functions under hypoxic conditions. The group showed that although the MEK inhibitor PD98059 (50 μmol/L) reduced HIF activity under hypoxic conditions, a highly selective and more potent MEK1/2 inhibitor, PD184352 (2 μmol/L), did not. This may be due to differences in the structure and mechanism of action of the two inhibitors and/or differences in the tumor model; the aforementioned study used osteosarcoma and breast adenocarcinoma models.

We then examined the hypothesis that AZD6244 could sensitize the tumor endothelium to the damaging effects of IR by affecting VEGF production. VEGF levels are increased by both HIF-1 (23) and MAPK signaling (19, 36), both of which have reduced activity in our model due to the activity of AZD6244 (Figs. 4 and 5). Not only is VEGF a survival factor that protects endothelial cells from the lethal effects of radiation (21, 22, 48), but a blockade of VEGF signaling may also induce normalization of the tumor vasculature leading to a window of opportunity whereby improved tumor oxygenation for radiotherapy efficacy is achieved (49). In vitro, AZD6244 reduced VEGF production by Calu-6 DC cells under hypoxic conditions, presumably by inhibiting the activity of both MEK and HIF-1 (Fig. 4C). In vivo, tumors grew rapidly after AZD6244 withdrawal, suggesting that viable tumor cells and vasculature remain following drug treatment alone (Fig. 2B). The fraction of vessels that were perfused was similar in control and AZD6244-treated tumors (65% versus 61%; Fig. 6A), suggesting that at the time assessed (after 10 days of treatment) at least, AZD6244 did not confer improved perfusion consistent with prolonged normalization.

The delayed regrowth of tumors treated with combined drug and radiation could be due to compromised tumor vessel functionality, a hypothesis supported by an observed reduction in tumor VEGF levels in response to AZD6244 treatment (Fig. 5C) and a marked lack of perfusion in the combination-treated tumors (Supplementary Fig. S2; Fig. 6A). Interestingly, prolonged exposure (10 days) to AZD6244 alone or in combination with radiotherapy decreased the level of binding of the hypoxic marker pimonidazole within the treated tumors. For the tumors treated with AZD6244 alone, the reduced hypoxic fraction was not associated with any changes in vascular parameters, perhaps suggesting that targeting MEK1/2 with this compound reduces oxygen consumption resulting in better overall oxygenation. In the combined treatment group, the lack of hypoxia was associated with an absence of perfusion. Although perhaps initially counterintuitive, this supports the pronounced vessel-targeting in the combination group. Consequently, pimonidazole delivery is severely compromised, and/or the tumor population has effectively undergone an ischemic cell death such that there are few hypoxic cells left that retain the capacity to undertake reductive metabolism of pimonidazole, thereby enabling the detection of hypoxic areas (Supplementary Fig. S2). Direct, real-time visual assessment of tumor vessels using xenografts grown in dorsal window chambers confirmed that the combination of AZD6244 and radiation had a significantly greater effect on the vasculature than treatment with either agent alone (Fig. 6C and D). This suggests that recovery of irradiated vasculature is impaired in the combination AZD6244 and radiation group. It is plausible that this effect contributed to the enhanced radiation response observed with both treatment schedules assessed in the Calu-6 DC studies. However, where radiotherapy was given during the last 5 days of AZD6244 treatment, the potential effect of having irradiated smaller tumors cannot be discounted.

In summary, the data presented support the use of the MEK1/2 inhibitor AZD6244 in combination with radiotherapy. A regimen combining AZD6244 and radiotherapy in Calu-6 and HCT116 tumor xenografts shows significantly increased antitumor effects compared with single therapy treatment. The underlying in vivo mechanisms may comprise direct radiosensitization of tumor cells by AZD6244 and modifications in tumor hypoxia coupled with drug-induced changes in HIF and VEGF activity that could sensitize both the tumor and its vasculature to radiation-induced damage. These data support the clinical development of AZD6244 in combination with radiotherapy and indicate a novel role for AZD6244 in inhibiting the tumor hypoxia response.

Disclosure of Potential Conflicts of Interest

P. Smith, A. Logie, R. Wilkinson, and S. Wedge are AstraZeneca employees. I. Stratford has received a commercial research grant and other commercial research support from AstraZeneca.

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