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The Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase Inhibitor AZD6244 (ARRY-142886) Induces Growth Arrest in Melanoma Cells and Tumor Regression When Combined with Docetaxel

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Abstract

Purpose: Disseminated melanoma is highly therapy resistant. The finding that 66% of melanomas harbor the activating BRAFV600E mutation has raised expectations for targeting the Ras/RAF/mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathway in melanoma. This study addresses the anti-melanoma activity of the MEK inhibitor AZD6244 (ARRY-142886).

Experimental Design: We recently have shown that growing melanoma cells as three-dimensional collagen-implanted spheroids enhances resistance to the MEK inhibitor U0126. Here, we investigated the anti-melanoma activity of AZD6244 in two-dimensional cell culture, the three-dimensional spheroid model, and an in vivo model.

Results: In two-dimensional cell culture, AZD6244 was cytostatic and reduced the growth of melanoma cells in a concentration-dependent fashion through the induction of G1-phase cell cycle arrest. In our three-dimensional spheroid model, the effects of AZD6244 were largely cytostatic and reversible, with drug washout leading to spheroid regrowth. Finally, 1205Lu cells were grown as tumor xenografts in severe combined immunodeficient mice. After tumor establishment, mice were dosed twice daily with 0, 10, or 30 mg/kg AZD6244 p.o. AZD6244 treatment decreased phospho-ERK in the tumors and significantly suppressed tumor growth. The original tumors remained viable, suggesting that AZD6244 monotherapy was largely cytostatic, and not proapoptotic in this model. Further studies showed that co-administration of AZD6244 (30 mg/kg) with docetaxel (15 mg/kg) led to tumor regression, indicating the potential for MEK inhibitor/chemotherapy drug combinations.

Conclusions: Inhibition of MEK is cytostatic as a monotherapy in melanoma, but cytotoxic when combined with docetaxel.

Melanoma is the most aggressive form of skin cancer and is highly resistant to conventional chemotherapy, immunotherapy, and targeted therapy. The prognosis for metastatic melanoma remains dismal with 5-year survival rates of 64% for patients with lymph node metastases and 16% for patients with distant metastases (1) and a median survival of 12 months and 4 to 6 months, respectively (2). Little progress has been made in the treatment of metastatic melanoma because of the absence of an effective systemic therapy. Thus, new therapeutic targets are urgently needed to improve the dismal prognosis of this disease.

In most melanomas, the mitogen-activated protein kinase (MAPK) pathway is constitutively active (3). This constitutive activity of the MAPK pathway arises through autocrine growth factor signaling through c-met and fibroblast growth factor 1R (4), αvβ3-integrin (3), or Notch1 (5) or through activating mutations in c-Kit (4%; ref. 6), Ras (15%; ref. 7), or BRAF (66%; ref. 8). In contrast, the MAPK pathway is not constitutively active in normal human melanocytes (9). These findings have raised expectations for targeted therapy in melanoma (8, 10, 11). The MAPK pathway consists of a cascade of three kinases that include the extracellular signal-regulated kinase (ERK1/2), which is phosphorylated and activated by the dual-specificity MAPK/ERK kinase (MEK1/2), which in turn is activated by a MEK kinase (MEKK, RAF; ref. 12). Once activated through RAF and MEK, ERK can migrate...
to the nucleus and drive cell proliferation through the activation of cyclin D1 and down-regulation of p27 (13). There is ample in vitro evidence that constitutive activity in the MAPK pathway plays a role in many other oncogenic processes of melanoma, such as invasion, escape from keratinocyte control, and immune system avoidance (14–18).

To date, the most intensively studied targeted therapy agent in melanoma is the multi-kinase inhibitor sorafenib (RAY 43-6009, Nexavar). Although this compound was initially thought to be a BRAF inhibitor, it actually has the most promising activity in renal cell carcinoma, which lacks BRAF mutations. In renal cell carcinoma, sorafenib is thought to work more through the suppression of tumor angiogenesis in part by inhibiting vascular endothelial growth factor receptor 2 [VEGFR2; kinase insert domain receptor (KDR); ref. 19].

A number of MEK inhibitors are currently undergoing preclinical and early clinical evaluation for a wide range of malignancies, including melanoma. Because these compounds lack the off-target effects of sorafenib, they are ideal for testing the viability of the MAPK pathway as a therapeutic target for melanoma. In the current study, we present the preclinical evaluation of AZD6244 (ARRY-142886, AstraZeneca), a highly selective allosteric inhibitor of MEK1/2; for its structure, see ref. 20. Through a series of mechanistic studies, we show that AZD6244 suppresses the growth of melanoma through the induction of cytostasis and has very little ability to induce apoptosis or block angiogenesis in these models.

Materials and Methods

Cell culture. The human melanoma cell lines WM35, WM793, 1205Lu, 451Lu, SbCl2, WM2032, WM1361a, WM852, and C8161 were cultured as described in ref. 21. The WM35, WM793, 1205Lu, and 451Lu cell lines were found to harbor the BRAFV600E mutation, whereas the SbCl2, WM2032, WM1361a, and WM852 harbored the Q61R mutation in N-Ras. The C8161 cell line is wild type for BRAF, and the N-Ras mutational status is unknown.

Comparative genomic hybridization and BRAF/N-Ras sequencing. Comparative genomic hybridization and mutation sequencing were done as described previously (22). The mutational status of WM35, WM793, 1205Lu, 451Lu, and SbCl2 was reported in ref. 23 and of C8161 in ref. 24.

Adherent cell proliferation analysis. Proliferation assays were done as described in ref. 25. Briefly, cells were plated into a 96-well plate containing 10% EMEM, 10% FBS, 1% L-glutamine, 1% Penicillin-Streptomycin, and 1% Glutamine. The resulting absorbance was read in a plate reader at 480 nm wavelength. Absorbance readings were subtracted from the value of blank wells, the reduction in cell growth was calculated as a percentage of control absorbance in the absence of any drug. Data show the viability of the MAPK pathway as a therapeutic target for melanomas, including melanoma, such as invasion, escape from keratinocyte control, and immune system avoidance (14–18).

Cell cycle analysis. Cell cycle analysis was done after treatment with kinase inhibitors (3 μmol/L, AZD6244; 10 μmol/L, U0126, for 24 h) and after incubation with kinase inhibitors for 24 h, followed by a further 24, 48, or 72 h without inhibitors as described in ref. 25. In other studies, melanoma cells were treated with either AZD6244 (3 μmol/L), docetaxel (10 nmol/L or 100 nmol/L; Hospital of the University of Pennsylvania Pharmacy), or a combination of AZD6244 and docetaxel for 24 h. Cells were harvested and analyzed as described above.

Three-dimensional spheroid growth. Melanoma spheroids were prepared using the liquid overlay method as described in ref. 25. After implantation into a gel of bovine collagen I containing EMEM, 1% L-glutamine, and 2% fetal bovine serum (FBS), spheroids were treated with AZD6244 (0.3–30 μmol/L), U0126 (0.3–30 μmol/L), docetaxel (10 nmol/L or 100 nmol/L) or AZD6244 (3 μmol/L) + docetaxel (10 nmol/L or 100 nmol/L) before being left to grow for 72 h. Spheroids were then washed twice in PBS before being treated with calcine-acetomethyl and ethidium bromide (Molecular Probes) for 1 h at 37°C according to the manufacturer’s instruction. After this time, pictures of the invading spheroids were taken using a Nikon-300 inverted fluorescence microscope.

Angiogenesis models. Morphogenesis assays on Matrigel were done as described in ref. 26. The media (DMEM/10% FBS) contained 0, 3, or 10 μmol/L AZD6244. As controls, serum-free media and media containing 10 μmol/L of the proteasome inhibitor MG-132 were used (27). Three-dimensional angiogenesis assays were done as described in ref. 28. The models were treated with AZD6244 (0, 3, or 10 μmol/L) for 2 × 72 h.

Immunofluorescence microscopy and terminal nucleotidyl transferase–mediated nick end labeling assay. Cultured cells were prepared, and immunofluorescence microscopy was done as described in ref. 25. Paraffin-embedded tissue sections were treated as described in refs. 29, 30. The BrdUrd staining was done as described in ref. 31. Immunofluorescence microscopy and terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assay using fluorescein-dUTP according to the manufacturer’s protocol (Roche Diagnostics).

In vivo experiments. The study protocol was approved by the Wistar Institute Animal Care and Use Committee (IAUC). Each group consisted of seven severe combined immunodeficient (SCID) CB-17 mice (Charles River Laboratories). Twenty-eight mice were injected s.c. with 1205Lu cells (2 × 106) into the lower back. When animals had developed melanoma nodules of about 5 mm in diameter, the study drug administration was initiated (day 1). The SCID mice were randomly assigned to four experimental groups of seven animals each: (a) no treatment at all; (b) 200 μL vehicle (0.5% hydroxypropyl methyl cellulose, Fluka) + 0.1% polysorbate (Tween 80, Fluka), sterilized by autoclaving at 121°C for 20 min at 30 psi (1.5 bar); (c) 10 mg/kg (in 200 μL vehicle); and (d) 30 mg/kg (in 200 μL vehicle) twice daily by oral gavage over a period of 14 days. The dose chosen in the present study was based on preliminary dose-finding experiments in SCID mice of up to 50 mg/kg AZD6244 twice daily (data not shown). It was noted that 30 and 50 mg/kg gave similar antitumor responses, and 30 mg/kg was chosen as the concentration for all subsequent experiments. At doses of 10 or 30 mg/kg twice daily, AZD6244 did not cause any apparent harm to the mice. In studies on hepatocellular carcinoma, it has recently been shown that up to 50 mg/kg AZD6244 were well tolerated by SCID mice (32). In other studies, mice were dosed thrice per week with 15 mg/kg docetaxel (Taxotere) by i.p. injection either
alone or in combination with twice daily AZD6244 (3 μmol/L) and U0126 (10 μmol/L) for 1 h. Blots were stripped and reprobed with an antibody against total ERK1/2 to confirm equal protein loading. In two-dimensional adherent cell culture, AZD6244 reduces the growth of melanoma cells in a concentration-dependent fashion. Adherent WM35, 451Lu, 1205Lu, or C8161 cells were treated with increasing concentrations U0126 (30 μmol/L to 3 μmol/L or 300 μmol/L to 30 μmol/L for C8161) or AZD6244 (300 μmol/L to 3 μmol/L or 3 nmol/L to 30 μmol/L for C8161) for 72 h before being treated with MT. The resulting changes in absorbance were read in a plate reader at 480 nm and expressed as a percentage of control absorbance. Data show the mean of six independent experiments ± SE.

Results

ERK1/2 is constitutively active in melanoma cell lines regardless of their BRAF status, which does not dictate the inhibition of pERK1/2 by the MEK1/2 inhibitors AZD6244 or U0126. A panel of 13 melanoma cell lines (five BRAFwt and eight BRAFV600E) was grown in two-dimensional adherent culture, and protein was extracted. Western blot analysis showed that ERK1/2 was constitutively active in these melanoma cell lines regardless of their BRAF status (a representative panel of nine cell lines is shown in Fig. 1A; see Materials and Methods for mutational status). Nine melanoma cell lines (five BRAFwt and four BRAFV600E) cultured adherently were treated with either 3 μmol/L AZD6244 or 10 μmol/L U0126 for 1 h. Both MEK1/2 inhibitors inactivated ERK1/2 in all cell lines, independent of their BRAF or N-Ras mutation status (Fig. 1A). To show dose and time dependence, similar experiments were done with two metastatic cell lines 1205Lu (BRAFV600E) and...
C8161 (BRAFwt) with increasing doses of AZD6244 (0, 0.0003, 0.003, 0.03, 0.3, and 30 μmol/L) for 1 h or at 3 μmol/L with increasing incubation times (0, 1, 4, 8, 24, 48, and 72 h). AZD6244 inhibited the ERK1/2 activity in 1205Lu cells completely at doses of 0.3 μmol/L and in C8161 cells at doses of 3 μmol/L (Supplementary Fig. S1). The complete inhibition lasted in 1205Lu cells for at least 48 h and in C8161 cells for at least 72 h (Supplementary Fig. S1). The inactivation of ERK

![Fig. 2.](image-url)
was associated with the up-regulation of p27 expression (data not shown).

In two-dimensional adherent cell culture, AZD6244 was cytostatic and reduced the growth of melanoma cells in a concentration-dependent fashion by causing G1-phase cell cycle arrest. Increasing concentrations of the MEK1/2 inhibitor AZD6244 reduced the growth of melanoma cell lines cultured as monolayer, but not of melanocytes or fibroblasts (a representative panel of four melanoma cell lines is shown in Fig. 1B). There was some selectivity for BRAF<sup>V600E</sup> over BRAF<sup>wt</sup> in two-dimensional cell growth inhibition. The IC<sub>50</sub> values for the BRAF<sup>V600E</sup> mutated melanoma cell lines were 4.1 nmol/L (WM35), 7.8 nmol/L (WM793), 4.9 nmol/L (1205Lu), and 8.4 nmol/L (451Lu). Values were not obtained for the melanoma cell lines without the BRAF<sup>V600E</sup> mutation because true growth inhibition plateaus were not reached. The MEK1/2

Fig. 3. AZD6244 treatment does not inhibit tube formation of HMVECs. A, ERK is active in adherent HMVECs and readily blocked by AZD6244. HMVECs were treated with DMSO (0) or 3 or 10 μmol/L AZD6244 for 1 h, total protein was extracted, and Western blots were done. Blots were stripped and reprobed with an antibody against total ERK1/2 to confirm equal protein loading. Untreated 1205Lu cells were used as a positive control for pERK. B, AZD6244 has a minor growth-inhibitory effect on HMVECs. HMVECs were treated with increasing concentrations of AZD6244 (300 pmol/L to 30 μmol/L) for 72 h before being treated with MTT. The resulting changes in absorbance were read in a plate reader at 480 nm and expressed as a percentage of control absorbance. Data show the mean of six independent experiments ± SE. C, HMVECs were grown on Matrigel for 8 h in DMEM containing 10% FBS in the presence of either DMSO (0) or 3 or 10 μmol/L AZD6244 or, as a negative control, in DMEM without FBS (starv). AZD6244 did not inhibit the formation of the capillary-like network. The graph shows the number of branchings of endothelial cells in the Matrigel assay, including the corresponding P values. AZD0, DMSO treated; AZD10, treated with 10 μmol/L AZD6244; starv, serum-starved negative control. Magnification ×10. D, HMVECs were grown in a collagen gel containing green fluorescent protein (GFP)–expressing fibroblasts (green) for 144 h in the presence of either DMSO (0) or 3 or 10 μmol/L AZD6244. The three-dimensional models were then fixed and incubated with an antibody against CD31 (red) and stained with DAPI (blue). Also in this model, AZD6244 treatment did not inhibit the tube formation of HMVECs. The graph shows the number of branchings of endothelial cells in the three-dimensional assay including the corresponding P values. AZD0, DMSO treated; AZD3, AZD10, treated with 3 or 10 μmol/L AZD6244, respectively. Magnification ×10.
inhibitor U0126 showed a similar growth inhibition, however, at approximately 70-fold higher concentrations (Fig. 1B, data not shown).

The growth arrest induced by AZD6244 was associated with the up-regulation of p27 expression and G1-phase cell cycle arrest, which was reversible upon removal of the drug regardless of their BRAF status (four BRAFV600E and three BRAFwt cells were investigated; representative data in Fig. 1C). Prolonged treatment (72 h) with AZD6244 led to some limited apoptosis in 1205Lu cells (BRAFV600E; Fig. 1C) but not in C8161 (BRAFwt) cells (Supplementary Fig. S2). The surviving BRAFV600E cells (Fig. 1C) as well as the BRAFwt cells (Supplementary Fig. S2) reentered S phase after removal of the drug. This was also confirmed by MTT assays, where the observed growth suppression was readily reversible upon AZD6244 washout in C8161 (BRAFwt, N-Raswt), but slower in 1205Lu cells (BRAFV600E; data not shown).

The effects of AZD6244 in three-dimensional melanoma spheroids are largely cytostatic and reversible, with drug washout leading to spheroid regrowth. We recently showed that culturing melanoma cells as three-dimensionally collagen-implanted spheroids enhances resistance to the MEK inhibitor U0126 (25). Whereas there is a homogeneous ERK1/2 activity in two-dimensional culture (Fig. 2A), in three-dimensional spheroids composed of C8161 cells (BRAFwt), ERK1/2 activity is mainly found in the growing periphery (Fig. 2C). Both in adherent cell culture and in the three-dimensional model, AZD6244 inhibits ERK1/2 activity (Fig. 2A-C). The collagen I gel that we used for the experiment is permeable for both AZD6244 and U0126 (25). Whereas there is a homogeneous ERK1/2 activity in two-dimensional culture (Fig. 2A), in three-dimensional spheroids composed of C8161 cells (BRAFwt), ERK1/2 activity is mainly found in the growing periphery (Fig. 2C). Both in adherent cell culture and in the three-dimensional model, AZD6244 inhibits ERK1/2 activity (Fig. 2A-C). The collagen I gel that we used for the experiment is permeable for both U0126 and AZD6244 (Fig. 2B). In the three-dimensional spheroid model, AZD6244 blocked the growth of the WM35 and WM793 cell lines (which are from radial growth phase and vertical growth phase stage melanomas, respectively) and had some minor growth-inhibitory activity against the 1205Lu and C8161 cell lines (both derived from metastases; Fig. 2D). AZD6244 is more potent than U0126 (25) at inhibiting cell growth and preventing the invasion of the tumor cells into the surrounding collagen. Like in two-dimensional adherent cell culture, the effects of AZD6244 in the three-dimensional tumor spheroid model are largely cytostatic and reversible, with drug washout leading to spheroid regrowth (data not shown).

AZD6244 treatment does not inhibit tube formation of human microvascular endothelial cells. The anti-melanoma effect of sorafenib in vivo has been shown to be related to its inhibition of the VEGFR and, therefore, suppression of angiogenesis (19). Therefore, we investigated the effect of AZD6244 on vessel morphogenesis by human microvascular endothelial cells (HMVEC). In adherently grown HMVECs, ERK is active and can be fully inhibited with AZD6244 (Fig. 3A). As compared with melanoma cells, AZD6244 has a minor growth-inhibitory effect on HMVECs (Fig. 3B). The in vitro ability of HMVECs to form capillaries was studied by the classic Matrigel assay (Fig. 3C). After 12 h, HMVECs spread throughout the Matrigel surface and aligned to form branching, anastomosing, and thick tubes with multicentric junctions, which gave rise to a closely knit network of capillary-like structures. Even doses of up to 10 μmol/l AZD6244 did not significantly inhibit the formation of this capillary-like network (Fig. 3C). We have recently developed a three-dimensional angiogenesis assay (28), which, in contrast to the Matrigel, also contains fibroblasts. Also, in this model, AZD6244 treatment did not significantly inhibit tube formation of HMVECs (Fig. 3D).

AZD6244 treatment significantly suppresses tumor growth and decreases proliferation and ERK activity but does not cause apoptosis in vivo. Next, we grew 1205Lu cells (BRAFV600E) as tumor xenografts in SCID mice. After tumor establishment (5 × 5 mm), mice were dosed twice daily with 0, 10, or 30 mg/kg AZD6244 by oral gavage. After 14 days, it was found that AZD6244 treatment had significantly suppressed tumor growth (tumor growth, nontreated: 9.47 ± 2.14-fold, treated with 30 mg/kg: 0.91 ± 0.10-fold; Fig. 4A and B) and decreased phospho-ERK in the tumors (Fig. 4C). Interestingly, the original tumors remained viable, again suggesting that AZD6244 is largely cytostatic and not proapoptotic in this model (Fig. 5A). Twenty-four hours before euthanasia, all mice...
were injected 1 mg BrdUrd. The BrdUrd uptake was decreased in mice treated with AZD6244, confirming the in vitro data suggesting that AZD6244 induces growth inhibition by inducing cell cycle arrest, but not apoptosis (Fig. 5B and C).

**Combined treatment of AZD6244 with docetaxel leads to enhanced in vitro apoptosis induction and some in vivo tumor regression.** Single-agent treatment of the melanoma cell lines used in this study with AZD6244 is antiproliferative but not cytotoxic. Because the end goal of clinical cancer therapy is tumor eradication, we tested AZD6244 in combination with the commonly used microtubule-stabilizing agent docetaxel (Taxotere). Treatment of the melanoma cells with docetaxel alone (10 and 100 nmol/L) for 24 h led to the expected profound G2-M phase cell cycle arrest (Fig. 6A), but little apoptosis. Combined treatment of the cells with AZD6244 (3 μmol/L) and docetaxel (10 or 100 nmol/L) for 24 h led to a combined G1 and G2-M phase arrest and an induction of apoptosis (Fig. 6A). The combined AZD6244 and docetaxel treatment also had significant antitumor activity in our three-dimensional spheroid model and was associated with greatly reduced sphere size and loss of cell viability, particularly when AZD6244 was combined with the higher concentration of docetaxel (Fig. 6B). As a final test, it was shown that co-administration of AZD6244 (30 mg/kg) twice daily and docetaxel (15 mg/kg) thrice a week led to a significant (P < 0.05) regression of the established tumor compared with AZD6244 (30 mg/kg) alone (Fig. 6C), suggesting the possible utility of combining MEK inhibitors with established chemotherapy agents. In contrast, administration of docetaxel alone had very little antitumor activity (Fig. 6C).

**Discussion**

Standard chemotherapy drugs have failed in clinical trials for melanoma. Novel approaches to melanoma therapy are therefore urgently needed. Recent work has focused on targeting signaling pathways that are known to be active in melanoma. Most interest has centered on the BRAF/MEK/ERK pathway. The role of constitutive MEK1/2 activity in melanoma is now well defined and includes increased cell proliferation, enhanced matrix metalloproteinase secretion, and invasion (11). The current study has investigated whether targeting MEK1/2 is a viable strategy for treating melanoma and has shown that, not only in vitro, but also in vivo, targeting MEK1/2 leads to growth inhibition through cytostasis.

Whereas ERK is not active in melanocytes (3), we showed here that ERK1/2 is constitutively active in melanoma cells regardless of their BRAF status. AZD6244 treatment reduced the growth of our panel of melanoma cell lines. In agreement with previously published studies, there was some selectivity of the compound for melanoma lines harboring the BRAF V600E mutation (10, 33). Cell lines with an N-Ras mutation were less sensitive to AZD6244, and it was not possible to obtain real IC50 values for these lines because a dose-response plateau was not reached.

Phosphorylated ERK is important for melanoma because it plays key roles in cell cycle entry, resistance of apoptosis, invasion, and possibly angiogenesis (11). Under physiologic conditions, cell cycle entry is regulated at the G1 restriction point. Cancer cells acquire the ability to overcome the G1 cell cycle checkpoint and enter S phase to replicate their DNA. Recent studies have shown that constitutively activated MEK1/2 and ERK are required for this transition in a variety of cell types (12). In melanoma cells, hyperactive ERK is required for transition to S phase because it is associated with increased cyclin D1 expression (11). In contrast, MEK1/2 activity is not required for growth of normal melanocytes (3). Therefore, the ability of melanoma cells to maintain ERK activity provides a potential therapeutic target for these cells.

Fig. 5. AZD6244-treated xenografts remain viable, suggesting that AZD6244 is largely cytostatic and not proapoptotic in vivo. One milligram of BrdUrd was injected i.p. 24 h before euthanasia. Immediately after euthanasia, xenografts were treated as mentioned above. A, TUNEL staining was done in paraffin-embedded sections (green; DAPI, blue). There is no increase in apoptotic cells in AZD6244-treated xenografts. B, Paraffin-embedded sections were coincubated with antibodies against BrdUrd (green) and S100 (red) and stained with DAPI (blue). There is a decrease in BrdUrd uptake after AZD6244 treatment (BrdU), confirming the in vitro data suggesting that AZD6244 induces growth inhibition by inducing cell cycle arrest, but not apoptosis. C, graph showing the percentage of BrdUrd-positive nuclei including corresponding P values.
cycle checkpoint, leading to uncontrolled growth. Progression through the G₁ restriction point into the S phase is driven by cyclin-dependent kinases (CDK) 4 and 6, which interact with cyclin D1, as well as by CDK2, which interacts with cyclins A/E (34). Constitutive activity in the MAPK pathway increases cyclin D1 and down-regulates p27 expression in melanoma cells (13) and is likely to be one mechanism that melanoma cells use to overcome the G₁ checkpoint. We showed here that Docetaxel treatment enhances the antitumor activity of AZD6244 in both *in vitro* and *in vivo* melanoma models. A, the combination of docetaxel and AZD6244 leads to enhanced apoptosis in melanoma cells. Adherent 1205Lu cells were treated with DMSO (control), AZD6244 (3 µmol/L), docetaxel (10 or 100 nmol/L) or AZD 6244 (3 µmol/L) + docetaxel (10 or 100 nmol/L) for 24 h. Cells were then fixed and stained with propidium iodide and analyzed using flow cytometry. Apoptosis is indicated by the increased proportion of cells in the sub-G₁ phase of the cell cycle. B, combining AZD6244 with docetaxel leads to reduced spheroid size and loss of cell viability. 1205Lu melanoma cells were grown under nonadherent conditions for 72 h until spheroids had formed. Spheroids were then harvested and implanted into a collagen gel before being treated with DMSO (0), AZD6244 (3 µmol/L), docetaxel (10 or 100 nmol/L), or AZD and docetaxel in combination. After 72 h, cells were treated with calcine AM, which stains living cells green, and ethidium bromide, which stains dead cells red. In all cases, data shown are representative of three independent experiments. Magnification, ×4. C, AZD6244 in combination with docetaxel (Taxotere) leads to some regression of 1205Lu melanoma cell xenografts. 1205Lu cells were grown as tumor xenografts in SCID mice. After tumor establishment (5 × 5 mm), mice were dosed daily with AZD6244 (30 mg/kg), docetaxel thrice a week (Taxotere, 15 mg/kg), a combination of AZD6244 (30 mg/kg) and docetaxel (15 mg/kg), or vehicle alone. Photographs of representative tumors for each group at day 14. Data show growth curves normalized to start volumes. *, the combination of AZD6244 and docetaxel led to a significant reduction in tumor volume compared with AZD6244 alone (*P < 0.05).
the inhibition of MEK1/2-ERK1/2 indeed is associated with the up-regulation of p27 expression and indeed causes G1 cell cycle arrest. Further support for a role of pERK in the progression of melanoma comes from the finding that higher pERK levels are found at the deeper margins of the melanoma where the tumor is invading into the dermis (35). With the predominance of pERK in the periphery of the spheroids as opposed to the homogenous distribution of pERK in adherent cells, we showed here for the first time that our three-dimensional model indeed mimics the in vivo situation better than the commonly used two-dimensional cultures. In some BRAFV600E cell lines, MEK1/2-inhibition leads to limited apoptosis. It has previously been shown that inhibiting the MAPK pathway is insufficient per se to effectively kill melanoma cells (33). In this study, we showed that invasion is strongly inhibited in earlier stage melanoma cells, but less so in metastatic cells (regardless of their BRAF status).

Previously, we showed that the additional inhibition of phosphoinositide-3-kinase may be a more effective strategy to inhibit invasion (25). Most studies thus far that have targeted BRAF/MEK signaling in melanoma were done using the multi-kinase inhibitor sorafenib. It is unclear whether the effects of sorafenib are a result of the inhibition of the MAPK pathway through the inhibition of BRAF. In xenograft models for renal cell carcinoma (RCC) that do not harbor the BRAFV600E mutation, treatment with sorafenib inhibits neither ERK phosphorylation nor Ki67 labeling. However, the treatment has significant inhibitory effects on tumor growth and vascularization and induces tumor apoptosis and hypoxia (19). These results suggest that the tumor growth-inhibitory effect of sorafenib in RCC is caused by the inhibition of VEGF$^1$–3 and platelet-derived growth factor-β receptors in endothelial cells, leading to inhibition of angiogenesis. Here, we show that despite ERK being active in endothelial cells and being readily blocked by AZD6244, there is only a minor effect on their proliferation. Moreover, using two different models, we showed that MEK1/2 inhibition with AZD6244 has no significant effect on angiogenesis, at least not by direct effects upon endothelial cells.

For the first time, we show here an inhibitor that directly targets the MAPK pathway in melanoma to correlate in vitro and in vivo data. The in vivo data show a striking cytostatic effect of AZD6244 on melanoma xenografts. AZD6244 fully inhibits growth at well-tolerated doses in vivo. Because AZD6244 is largely cytostatic in these models of melanoma, it is important to define effective drug combination partners that lead to tumor regression. Here, we have shown that the co-administration of AZD6244 with a commonly used taxane, docetaxel, leads to a reduction in the size of the established melanoma xenografts. Interestingly, this mirrors some of the recent clinical success of sorafenib in melanoma, where responses to sorafenib are only seen in combination with carboplatin and paclitaxel. Currently, the mechanism of interaction between MEK inhibitors and taxanes is unknown. There is, however, some suggestion that MEK/ERK activity is required for the successful execution of mitosis, and that the combination of MEK inhibitors and taxanes increases the level of mitotic catastrophe (36). It is clear that the strong addiction of melanomas to BRAF/MEK/ERK activity makes MEK inhibitors an attractive option for any future optimized drug regimen for this most deadly of tumors.

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