Basal and Treatment-Induced Activation of AKT Mediates Resistance to Cell Death by AZD6244 (ARRY-142886) in Braf-Mutant Human Cutaneous Melanoma Cells

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Abstract

The majority of melanomas show constitutive activation of the RAS-RAF-MAP/ERK kinase (MEK)-mitogen-activated protein kinase (MAPK) pathway. AZD6244 is a selective MEK1/2 inhibitor that markedly reduces tumor P-MAPK levels, but it produces few clinical responses in melanoma patients. An improved understanding of the determinants of resistance to AZD6244 may lead to improved patient selection and effective combinatorial approaches. The effects of AZD6244 on cell growth and survival were tested in a total of 14 Braf-mutant and 3 wild-type human cutaneous melanoma cell lines. Quantitative assessment of phosphoprotein levels in the Braf-mutant cell lines by reverse phase protein array (RPPA) analysis showed no significant association between P-MEK or P-MAPK levels and AZD6244 sensitivity, but activation-specific markers in the phosphoinositide 3-kinase (PI3K)-AKT pathway correlated with resistance. We also identified resistant cell lines without basal activation of the PI3K-AKT pathway. RPPA characterization of the time-dependent changes in signaling pathways revealed that AZD6244 produced durable and potent inhibition of P-MAPK in sensitive and resistant Braf-mutant cell lines, but several resistant lines showed AZD6244-induced activation of AKT. In contrast, sensitive cell lines showed AZD6244 treatment–induced upregulation of PTEN protein and mRNA expression. Inhibition of AKT, TORC1/2, or insulin-like growth factor I receptor blocked AZD6244-induced activation of AKT and resulted in synergistic cell killing with AZD6244. These findings identify basal and treatment-induced regulation of the PI3K-AKT pathway as a critical regulator of AZD6244 sensitivity in Braf-mutant cutaneous melanoma cells and the novel regulation of PTEN expression by AZD6244 in sensitive cells, and suggest new combinatorial approaches for patients.

Introduction

Melanoma is the most aggressive form of skin cancer. Patients with metastatic disease have a median survival of <1 year, and outcomes are not improved with chemotherapy or immunotherapy (1). A review of the Surveillance Epidemiology and End Results database from 1950 to 2000 showed that the incidence of and mortality from melanoma are increasing at a greater rate than for any other cancer. Thus, there is a tremendous need for more effective therapeutic approaches for this disease. The majority of melanomas harbor an activating mutation in the RAS-RAF-mitogen-activated protein kinase (MAPK) signaling pathway. Braf mutations are detected in approximately 50% of melanomas (2). Over 90% of these mutations affect the V600 residue, and they result in constitutive activation of the BRAF protein (3). In addition, approximately 20% of melanomas have an activating Nras mutation (2). Mutations in Nras also activate the RAS-RAF-MAPK pathway (4). As Braf and Nras mutations are mutually exclusive (5, 6), approximately 70% of melanomas harbor a mutation in the RAS-RAF-MAPK pathway. Many melanomas without identifiable mutations in the pathway also show constitutive MAPK activation (7). Thus, activation of the RAS-RAF-MAPK pathway is likely a critical event in melanoma, and it may be an important therapeutic target.

AZD6244 (ARRY-142996, Selumetinib) is a potent, highly selective, uncompetitive inhibitor of MAP/ERK kinase 1/2 (MEK1/2; ref. 8). AZD6244 has an in vitro IC₅₀ of 14 nmol/L against purified MEK, whereas minimal inhibition was seen in >40 kinases at 10 μmol/L. AZD6244 inhibited extracellular signal-regulated kinase (ERK) phosphorylation (P-ERK) at ~40 nmol/L in cells growing in vitro and in xenografts growing in mice. Initial testing showed that many different cancer types, including melanoma, colon, breast, and pancreatic cancer with Braf or Nras mutations, were sensitive to the...
inhibitory effects of AZD6244, although the degree of growth suppression varied even among cell lines with those mutations (9). Phase I clinical testing in patients showed that AZD6244 was well tolerated (10). Paired biopsies from 20 patients with assessable tissue showed a mean reduction in tumor P-ERK of 79% with AZD6244 treatment. A subsequent randomized phase II trial in metastatic melanoma compared AZD6244 with temozolomide, a standard cytotoxic agent used in melanoma (11). Even among the patients with Braf mutations (n = 67), which in vitro generally correlated with sensitivity to AZD6244, surprisingly there was only a 12% clinical response rate, which was not superior to temozolomide.

The clinical experience with AZD6244 shows that doses can be achieved in patients that significantly inhibit the RAS-RAF-MAPK pathway. There is also evidence that clinical responses can be achieved in some patients. However, the rate of clinical response, particularly among Braf-mutant patients, is lower than predicted by preclinical testing and less than that reported with the RAF inhibitor PLX4032/RG7204 (12). The future clinical use of AZD6244 would be enhanced by an improved understanding of the factors that predict and regulate sensitivity to the drug. Previous testing of other MEK inhibitors in melanoma cell lines showed that P-ERK levels, both at baseline and with treatment, did not correlate with the degree of cell growth inhibition that was achieved (13). Studies are currently ongoing to further investigate a possible threshold level of in vivo pathway inhibition that correlates with clinical responses to RAS-RAF-MAPK pathway inhibitors, particularly PLX4032. However, studies with other targeted therapies have shown that inhibition of the intended target often results in unexpected activation of other pathways through feedback regulation of signaling networks (14, 15). Understanding these changes can identify combinatorial approaches that overcome these effects and improve efficacy. We have developed reverse phase protein arrays (RPPA) to perform efficient and quantitative assessment of signaling pathways in cancer (16). We have used RPPA to perform integrated analysis of kinase signaling pathways with clinical and molecular features in a number of cancers, including melanoma (17–19). We have also used RPPA to identify time- and dose-dependent changes in kinase signaling networks in cancer cells in response to growth factors and targeted therapies (20, 21).

To improve our understanding of the regulators of treatment response in melanoma, we analyzed a panel of Braf-mutant human cutaneous melanoma cell lines for their sensitivity to growth and survival inhibition by AZD6244. We compared these effects with the baseline activation status of signaling pathways in the cells, and with AZD6244 treatment–induced changes in signaling networks. These studies have identified the phosphoinositide 3-kinase (PI3K)-AKT pathway as a critical regulator of the efficacy of AZD6244 in Braf-mutant melanomas, including in cells with baseline activation of the pathway. We also identified and validated combinatorial approaches that increase the cytoidal effects of AZD6244 in Braf-mutant melanoma cell lines, which suggest strategies for testing in patients.

Materials and Methods

Cell lines and inhibitors
All human melanoma cell lines were authenticated by short tandem repeat fingerprinting, as previously described (17). The mutation status of Braf, Nras, c-Kit, PIK3CA, and Akt1/2/3 was determined by mass spectroscopy–based genotyping, and has been reported previously (17, 22, 23). Cells were maintained in RPMI media in 5% fetal bovine serum (Gemini Bioproducts) at 37°C supplemented with 5% CO2. AZD6244 and AZD8055 were obtained under a material transfer agreement with Astra Zeneca. Rapamycin was from Cell Signaling Technology. Recombinant insulin-like growth factor I (IGF-I) was from Invitrogen.

Cell viability assay
Cells were seeded in 96-well plates overnight and treated with increasing concentrations of drugs or DMSO (vehicle). The DMSO concentrations were maintained at 0.02% in all wells. After 48-hour incubation, cell viability was determined using Cell Titer Blue Cell Viability Assay (Promega). Data were analyzed and graphed using MS-Excel.

Cell cycle analysis
Cells were seeded in 6-well plates were treated with inhibitors and/or siRNA as described. After the indicated treatment periods, cells were trypsinized, fixed in 70% ethanol, and stained with propidium iodide (BD Biosciences). Cell cycle analyses were performed as described. Apoptosis was determined by the cytoplasmic histone-associated DNA fragment method using the Cell Death Detection ELISA Plus Kit (Roche Applied Science).

RPPA and Western blotting analysis
Cells were seeded in 100-mm tissue culture plates overnight, treated with AZD6244 or vehicle (DMSO) in triplicate, and harvested at the indicated time points. Protein lysate preparation for RPPA analysis has been described previously (16–20). RPPA analysis was performed by the University of Texas M.D. Anderson Functional Proteomics Core Facility. Samples were analyzed for the expression of 94 protein markers using RPPA-validated antibodies (Supplementary Table S1). Results were normalized as previously described (17), and were reported as log2 values. After correcting for sample loading differences, for each cell line and time point the protein expression levels were normalized against those of DMSO-treated cells. Clustering analysis was performed using Cluster 2.1 and was visualized using Treeview software (http://rana.lbl.gov/EisenSoftware.html). Western blotting analysis was performed using lysates prepared through the same methods, using standard Western blotting procedures (16). Antibodies...
used for Western blotting are also listed in Supplementary Table S1. Those not listed in the table are GAPDH (Ambion), FOXO3A, P-FOXO3A (S318), IGF-I receptor (IGFIR), P-IGFIR (Y1131), and P-IGFIR (Y1135; Cell Signaling Technology).

siRNA transfections

Cells seeded in 6-well plates were transfected with 20 nmol/L of siRNA, using XtremeGene (Roche Applied Science) or Dharmafect 1 (Dharmacon) transfection reagents. Inhibitors were

Figure 1. Growth and survival inhibition by AZD6244. A, growth inhibition in human melanoma cell lines treated with AZD6244 for 48 hours. X-axis, concentration of AZD6244 (nmol/L); Y-axis, % growth inhibition. 1, cells with high sensitivity; 2, cells with moderate sensitivity; 3, cells with low sensitivity. Cells with data points connected by dotted lines are wild-type for Braf; all other cell lines have Braf mutations. Data points, average of three replicates; error bars, SD. B, cell cycle analysis of WM35 cells (group 1) after 72-hour AZD6244 treatment. X-axis, concentration of AZD6244 (nmol/L); Y-axis, % of cell population. Black, dark gray, light gray, and white bars are the sub-G1 (dead cells), G1, S, and G2-M phases of the cell cycle. Each bar, average of two replicates; error bars, SD. C, cell cycle analysis of SKMEL5 cells (group 2). D, cell cycle analysis of HS294T cells (group 3).
added after 48 hours, and cells were harvested after 24 hours or 48 hours for Western blotting and cell cycle analysis, respectively. The siRNAs used were siControl RISC-free, siPTEN, siAKT (mix of AKT1, AKT2, and AKT3 siRNAs), siIGFIR, (Dharmacon), and siIGFII (Ambion).

**RNA analysis**

Cells seeded in 10-cm plates were treated with AZD6244 or vehicle (DMSO). After 24-hour incubation, RNA was isolated using the miRNeasy kit (Qiagen). RNA was quantified using Nanodrop. Reverse transcription and PCR amplification were performed using the Taqman One-Step RT-PCR assay reagent using PTEN or GAPDH primer-probe mixes from Applied Biosystems Inc. Fold changes in the treated samples compared with controls were determined using the formula: fold change = efficiency^ΔΔCt, where ΔΔCt is the threshold cycle difference between control and treated samples normalized against GAPDH expression.

**Measurement of secreted IGF-I**

Cells (10^5) were seeded in 6-well plates overnight, replenished with 0.5 mL fresh media, and treated with AZD6244 for 24 hours. siRNA-treated cells were replenished with 0.5 mL of fresh media 48 hours after transfection and then were treated with AZD6244 for 24 hours. Secreted IGF-I in the media was quantified using the Quantikine human IGF-1 Immunoassay kit (R&D Systems).

**Results**

**Comparison of baseline signaling pathways with growth inhibitory effects of AZD6244**

We initially determined the relative sensitivity of 11 Braf-mutant cutaneous melanoma cell lines to growth inhibition by AZD6244 (Fig. 1A). This panel included four cell lines (WM2664, WM1799, UCSD-354L, WM1552) with concurrent PTEN loss and one cell line with concurrent Akt3 E17K mutation (WM46). We also tested two cell lines that were wild type for Braf and Nras (Mewo, HS294T). Overall, the cell lines were organized in three groups, which we called group 1 (high sensitivity), group 2 (intermediate sensitivity), and group 3 (low sensitivity). Of the wild-type cell lines, HS294T was not sensitive to AZD6244 (group 3), but Mewo was (group 1). To further investigate the observed heterogeneity in sensitivity to AZD6244, cell cycle analysis was performed on cell lines from each group. The group 1 cell lines WM35 (Fig. 1B), A375, and UACC257 (Supplementary Fig. S1) showed a G1 cell cycle arrest with 40 nmol/L AZD6244, and a marked sub-G1 accumulation with higher doses (i.e. >60% sub-G1 with 360 nmol/L). AZD6244 treatment of the group 2 cell lines SKMEL5 (Fig. 1C), MEL624, UCSD354L, and WM1799 (Supplementary Fig. S1) induced G1 cell cycle arrest, but no significant cell death (sub-G1) was observed up to 3 μmol/L. The highly resistant group 3 HS294T cells (Fig. 1D) did not undergo significant G0 or sub-G1 accumulation up to 1 μmol/L AZD6244 (Fig. 1D). Representative cell cycle profile histograms of AZD6244-treated cells belonging to each group are shown in Supplementary Fig. S2. A cytoplasmic histone-DNA-fragment-based apoptosis assay confirmed that the group 1 cells underwent apoptosis, whereas the group 2 and group 3 cells did not (Supplementary Fig. S3). Time-course cell cycle analysis of the sensitive cell lines found that cell death was observed after 48 hours with AZD6244 treatment, whereas resistant cells showed no significant cell death for at least 72 hours (Supplementary Fig. S4). Drug washout experiments with WM35 and A375 showed that drug exposure of 24 to 48 hours was required to induce cell death (data not shown).

To determine if activation of specific protein signaling networks correlated with resistance to AZD6244 in the Braf-mutant cell lines, we compared the IC50 data (Supplementary Table S2) for the cell lines with the expression of 99 protein expression levels were determined for the cell lines growing under normal tissue culture conditions by RPPA. r^2, Pearson correlation. P was determined by the t-statistic.

**Table 1. The 10 proteins with the highest correlation with resistance (increased IC50) to AZD6244 in Braf-mutant human melanoma cell lines**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein expression vs. IC50 (r^2)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>P-AKT_Thr308</td>
<td>0.65</td>
<td>0.02</td>
</tr>
<tr>
<td>C-RAF</td>
<td>0.55</td>
<td>0.06</td>
</tr>
<tr>
<td>IGFIR β</td>
<td>0.55</td>
<td>0.07</td>
</tr>
<tr>
<td>P-AKT_Ser473</td>
<td>0.53</td>
<td>0.08</td>
</tr>
<tr>
<td>eIF4E</td>
<td>0.51</td>
<td>0.09</td>
</tr>
<tr>
<td>P-TSC2_Thr1462</td>
<td>0.50</td>
<td>0.10</td>
</tr>
<tr>
<td>P27</td>
<td>0.43</td>
<td>0.16</td>
</tr>
<tr>
<td>P-GSK3α/β_Ser21/9</td>
<td>0.42</td>
<td>0.18</td>
</tr>
<tr>
<td>Cycin E</td>
<td>0.41</td>
<td>0.19</td>
</tr>
<tr>
<td>AMPK</td>
<td>0.40</td>
<td>0.20</td>
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NOTE: Protein expression levels were determined for the cell lines growing under normal tissue culture conditions by RPPA. r^2, Pearson correlation. P was determined by the t-statistic.
Treatment-induced changes in signaling pathways by AZD6244

To identify early and late signaling events that correlate with cell death induction by AZD6244, we performed RPPA analysis on A375 and WM35 cells treated with 360 nmol/L AZD6244 for 0.5, 3, 6, 12, 24, and 48 hours. Identical experiments were performed with SKMEL5 and MEL624 cells, which undergo G1 phase growth arrest only in response to AZD6244, and the highly resistant HS294T cells, which do not die or arrest. Triplicate protein lysates were analyzed, and protein expression levels for each cell line were normalized to DMSO-treated controls.

We initially analyzed the effects of AZD6244 on the expression of P-P44/42 MAPK. We observed a similar degree (≥90%) and duration (≥48 hours) of inhibition of P44/42 MAPK phosphorylation in the sensitive and resistant Braf-mutant cell lines (Supplementary Fig. S5). The HS294T cells had much lower baseline levels of P-MAPK, but this activity was inhibited by treatment with AZD6244, suggesting that their resistance was not due to lack of intracellular drug accumulation (Supplementary Fig. S5E–F). To analyze the role of other pathways, we performed hierarchical clustering analysis (by time) of the RPPA data for each of the cell lines (Fig. 2). Comparison with the completely resistant HS294T cells identified a group of proteins that were similarly decreased in the other four cell lines, including P-MAPK, P-YB1, cyclin D1, and P-S6. The HS294T cells showed a marked increase in P-MEK levels upon treatment with AZD6244, which was unique compared with the other cell lines.

Patterns of protein expression in the group 1 versus the group 2 cells were compared to identify signaling events that might determine cell death induction versus cytostasis with AZD6244. The MEL624 and SKMEL5 cells, which undergo G1 arrest but not cell death with AZD6244, showed a time-dependent increase in the expression of P-AKT_Ser473 and P-AKT_Thr308 that was not observed in the WM35 or A375 cells (Fig. 2). In contrast, the WM35 and A375 cells showed upregulation of PTEN protein expression, as well as cleaved caspase 7 (apoptosis marker), and p27. Western blotting confirmed the time-dependent increase in P-AKT in the moderately resistant SKMEL5 and MEL624 (Fig. 3A). In contrast, the highly sensitive WM35, A375 (Fig. 3A), and UACC257 (Supplementary Fig. S6) showed no induction of P-AKT. WM35 cells showed a marked increase in PTEN levels, which was also detected, but was less dramatic, in A375 and UACC257 cells. To determine if these effects were specific to these cells, Western blotting was performed on additional cells recently identified to be resistant to AZD6244 (25). The Braf/Nras-wild-type D24 cells, which were similar to group 2 cells in their relative sensitivity to growth inhibition and G1 cell cycle arrest (Supplementary Fig. S7), showed increased P-AKT with AZD6244 treatment (Supplementary Fig. S6). The Braf-mutant PTEN-expressing D29 cells, which by growth inhibition and cell cycle were similar to the HS294T cells (Supplementary Fig. S7), did not show increased P-AKT, but did have increased P-MEK (Supplementary Fig. S8). The Braf-mutant PTEN-expressing
MM595 and A2 cells also were similar to HS294T in their growth resistance (Supplementary Fig. S7), but they did not show an increase in P-AKT or P-MEK (Supplementary Fig. S8).

We further investigated the observed increase in PTEN protein expression in the sensitive cell lines. Reverse transcriptase-PCR analysis showed a >6-fold increase in PTEN transcript levels in the WM35 and A375 cells following AZD6244 treatment, whereas no significant change was observed in the SKMEL5, MEL624, or HS294T cells (Fig. 3B). To test the functional significance of PTEN induction, WM35 cells were transfected with siRNA against PTEN. Knockdown of PTEN increased the basal levels of P-AKT and reduced the AZD6244-induced cleavage of caspase 7 (Fig. 3C). PTEN knockdown also reduced AZD6244-induced cell killing as measured by both fluorescence activated cell sorting (FACS) analysis (Fig. 3D) and apoptosis assay (Fig. 3E).

**Inhibition of the PI3K-AKT pathway sensitizes melanoma cells to AZD6244**

To test if activation of AKT mediated resistance to AZD6244-induced cell death, SKMEL5 cells were transfected with siRNA against AKT. Greater than 90% knockdown of AKT was achieved; decreased expression of P-GSK3α/β and P-FOXO3a confirmed functional inhibition of AKT activity (Fig. 4A). Compared with transfection with control siRNA, knockdown of AKT resulted in a slight increase in cell death (12.5% versus 3.3%), as assessed by FACS analysis (Fig. 4B). Knockdown of AKT markedly sensitized the SKMEL5 to

**Figure 3.** PTEN expression and function in AZD6244-mediated cell death. A, Western blotting analysis of group 1 (sensitive) WM35 and A375 (top) and group 2 (resistant) MEL624 and SKMEL5 (bottom) following treatment with AZD6244. Time point is above the P-AKT results (hours). Cl. casp. 7, cleaved caspase 7. B, PTEN mRNA expression following AZD6244 treatment. Fold change versus baseline in PTEN mRNA as measured by quantitative reverse transcriptase-PCR after 24-hour treatment with 360 nmol/L of AZD6244. X-axis, cell line; Y-axis, relative fold change in PTEN transcripts. Bars, average of three samples; error bars, SD.
AZD6244-induced cell death, with 40% sub-G1 accumulation as compared with 5% with AZD6244 alone (Fig. 4B), which was confirmed as apoptosis (Fig. 4C).

Previous studies in human breast cancer cell lines indicated that activation of AKT following MEK inhibition may be mediated through the epidermal growth factor receptor (EGFR; ref. 14). Like most melanomas, however, both the SKMEL5 and MEL624 do not express detectable EGFR protein by RPPA (data not shown). IGF-I and its receptor (IGFIR) have been shown to play an important role in promoting melanoma growth through activation of the PI3K-AKT pathway (26–28). AZD6244-resistant cells showed high correlation (Pearson $r = 0.55$, $P = 0.07$) with basal levels of IGFIR protein in the RPPA analysis (Table 1) and Western blotting (Supplementary Fig. S9). The levels of IGFIR protein were the highest in the group 2 cells MEL624 and SKMEL5, when compared with the other melanoma cell lines (Supplementary Fig. S9). Knockdown of IGFIR expression by siRNA abrogated AZD6244-induced P-AKT (Supplementary Fig. S10). Conversely, treatment of the sensitive WM35 cells with recombinant IGF-I induced AKT activation (Supplementary Fig. S11A) and reduced AZD6244-induced apoptosis, but it did not alter the induction of a G1-phase arrest (Fig. 5 and Supplementary Fig. S11B). This suggests that IGFIR signaling is a critical determinant of AZD6244-induced cell death, but not the cytostatic effects of the drug. Of note, we did not detect changes in the total IGFIR levels or phosphorylation of IGFIR at residues Y1131 and Y1135 following AZD6244 treatment (data not shown), but this does not preclude the possibility of differential effects on other phosphorylation sites or other regulators of this pathway.

AZD8055 is a novel mammalian target of rapamycin (mTOR) kinase inhibitor that inhibits the activity of both the mTORC1 and mTORC2 complexes and therefore inhibits AKT activation (29). Treatment of SKMEL5 cells with AZD8055 inhibited the AZD6244-induced increase in IGF-I growth factor secretion into the medium by the moderately resistant cells, and knockdown of IGF-I by siRNA blocked AZD6244 treatment-induced increase in P-AKT (Supplementary Fig. S10). Conversely, treatment of sensitive WM35 cells with recombinant IGF-I induced AKT activation (Supplementary Fig. S11A) and reduced AZD6244-induced apoptosis, but it did not alter the induction of a G1-phase arrest (Fig. 5 and Supplementary Fig. S11B). This suggests that IGFIR signaling is a critical determinant of AZD6244-induced cell death, but not the cytostatic effects of the drug. Of note, we did not detect changes in the total IGFIR levels or phosphorylation of IGFIR at residues Y1131 and Y1135 following AZD6244 treatment (data not shown), but this does not preclude the possibility of differential effects on other phosphorylation sites or other regulators of this pathway.

AZD8055 is a novel mammalian target of rapamycin (mTOR) kinase inhibitor that inhibits the activity of both the mTORC1 and mTORC2 complexes and therefore inhibits AKT activation (29). Treatment of SKMEL5 cells with AZD8055 inhibited the AZD6244-induced increase in
P-AKT (S473), whereas P-AKT (T308) was mostly unchanged (Fig. 6A). Treatment with rapamycin, which inhibits the mTORC1 but not the mTORC2 complex, resulted in increased P-AKT at both residues. FACS cell cycle analysis showed that treatment of SKMEL5 cells with AZD8055 alone did not result in significant cell death, but it did when combined with AZD6244 (Fig. 6B). Rapamycin did not induce cell death, either as a single agent or when combined with AZD6244. Similar results were observed with the apoptosis assay (Supplementary Fig. S12A). Treatment of MEL624 cells with AZD8055 also inhibited P-AKT (S473) expression, whereas the levels of P-AKT (T308) were slightly increased (Fig. 6C). FACS analysis (Fig. 6D) and apoptosis assay (Supplementary Fig. S12-B) both showed cell death induction following combined treatment with AZD6244 and AZD8055.

Discussion

The RAS-RAF-MAPK signaling pathway is an attractive target for therapy development for melanoma due to the high prevalence of activation of this pathway. Although the potent inhibition of this pathway has been shown, AZD6244 had low activity as a single agent in melanoma, even in patients with \textit{Braf} mutations. As \textit{Braf} mutations are the most common activating mutation in cutaneous melanomas, and as most evidence supports that they primarily act through the activation of MEK-MAPK signaling, we performed a proteomic analysis to explore the role of kinase signaling pathways in resistance to AZD6244.

Initial testing of the effects of AZD6244 on growth and survival segregated the \textit{Braf}-mutant melanoma cell lines into two groups: group 1 showed marked growth inhibition and significant cell death induction with nanomolar concentrations of AZD6244, whereas group 2 was less growth inhibited and exhibited G1 cell cycle arrest but no significant cell death. We also identified cells (HS294T) that were not growth inhibited significantly by AZD6244 up to micromolar doses, which were therefore used as a model for effects of AZD6244 in the absence of any growth inhibitory effects. Previously, we had performed RPPA analysis of melanoma cell lines for a variety of proteins in signaling pathways (17). Analysis of basal protein expression levels identified weak correlation between levels of P-MEK or P-44/42 MAPK and AZD6244 IC\textsubscript{50} values, similar to previous experiments in melanoma cell lines with the MEK inhibitor U0126 (13). In contrast, a number of activation-specific markers of the PI3K-AKT pathway correlated with resistance to AZD6244. Although this study is the first to report a significant correlation of increased PI3K-AKT activity with resistance to MEK inhibitors in melanoma, these results mirror the results recently reported in a study of AZD6244 in lung cancer cell lines (30). We also observed a positive correlation for CRAF expression and AZD6244 IC\textsubscript{50} values, although it did not quite reach statistical significance (Table 1). Increased CRAF expression has also been associated with resistance to BRAF inhibitors (31).

It is not surprising that \textit{Braf}-mutant melanoma cells with concurrent activation of the PI3K-AKT pathway have increased resistance to the growth inhibitory effects of MEK inhibition. Although the clinical trials with AZD6244 have not reported an analysis of the activation status of the PI3K-AKT pathway in treated patients, we and others have shown that genetic mutations and constitutive activation of the PI3K-AKT pathway are present in only a subset of \textit{Braf}-mutant melanomas (17, 23, 32–35). Thus, basal-activation of the PI3K-AKT pathway is likely not sufficient to fully explain or predict resistance to AZD6244 in melanoma. We noted that
although the resistant group 2 included two cell lines with PTEN loss and very high levels of P-AKT, it also included two cell lines (SKMEL5, MEL624) with normal PTEN and low basal P-AKT. We therefore performed further testing to determine possible mechanisms of resistance in these cell lines by comparing the effects of AZD6244 treatment on their signaling pathways with effects in sensitive cell lines. Although all four of these Braf-mutant cell lines showed similar degree and duration of MAPK inhibition and several other proteins, the resistant cell lines increased their P-AKT levels following exposure to AZD6244, which was not observed in the sensitive cell lines. The functional significance of AKT activation is supported by the fact that inhibition of AKT activity, either by AKT knockdown or concurrent treatment with the mTORC1/2 inhibitor AZD8055, resulted in synergistic cell killing in the resistant cell lines. These findings give support for the upcoming clinical trial testing the effects of AZD6244 and MK2206 (www.clinicaltrials.gov), a small molecule inhibitor of AKT, as well as further exploration of the effects of AZD6244 and AZD8055. We also tested signaling effects in additional cell lines recently characterized for resistance to AZD6244 by another laboratory (25). We found that several of these cells lines exhibited similar effects to those observed in our panel. However, the differential results in the MM595 and A2 cells support the likely existence of additional resistance mechanisms.

Previous studies in breast cancer cell lines showed that MEK inhibition resulted in cross-activation of the EGFR tyrosine growth factor receptor (36). As this receptor is not frequently expressed in melanoma or in the cell lines used in this experiment, we hypothesized that other growth factor receptors could mediate this effect. We tested the role of the IGF-I pathway, as it previously has been implicated in AKT activation in melanoma (26–28). Inhibition of IGFIR

Figure 5. Effects of IGFIR signaling on AZD6244-induced activation of AKT. A, Western blotting of SKMEL5 cells transfected with RISC-free or IGFIR siRNA. After 48 hours cells were treated with AZD6244 360 nmol/L or vehicle, then were harvested after 24 hours. B, FACS analysis and apoptosis induction (C) of SKMEL5 cells transfected as in A. Cells were harvested for analysis after 48 hours of drug exposure. D, effect of IGF-I on survival of AZD6244-treated WM35 cells. WM35 cells were treated with vehicle or AZD6244 360 nmol/L, in the absence or presence of 100 ng/mL IGF-I. Cells were harvested after 48 hours, and FACS analysis was performed. Bars, average of triplicates; error bars, SD.
did not kill SKMEL5 cells, but it blocked the induction of P-AKT by AZD6244 and induced cell death in combination with AZD6244. AZD6244 treatment induced a slight increase of IGF-I secretion by the cells, and knockdown of IGF-I also blocked P-AKT induction by AZD6244. Supporting a specific role for the pathway in cell survival, recombinant IGF-I treatment blocked AZD6244-induced cell death, but not growth arrest, in the sensitive WM35. These results support the need for further testing the effects of combining AZD6244 with inhibition of IGFIR-mediated signaling. Further work is also merited to better understand the mechanism of cross-talk to this pathway, to help identify and assess optimal combinatorial approaches. Additional testing will also be needed to determine if this combinatorial approach is most effective in melanomas with elevated IGFIR expression.

Our finding that MEK inhibitor treatment can induce the upregulation of PTEN transcript and protein is unprecedented. The mechanism(s) of the transcriptional regulation of PTEN remains poorly understood. Although several microRNAs (miRNAs) have been shown to regulate PTEN transcript stability (37–39), our profiling of eight miRNAs that recognize sites in the PTEN message (miR-10a, -26a, -29a, -30a-3p, -30c-3p, -183, -221, and -519d) did not show changes or differential effects that would explain the observed differences in PTEN expression (data not shown). Understanding these effects may require more comprehensive miRNA profiling, or functional analysis of the PTEN promoter. Identification of molecular features that predict the upregulation of PTEN expression with MEK inhibition may indicate patients who would selectively benefit from treatment with these agents. Such markers would need to be validated in larger panels of cell lines and patients.

These studies have provided new insights into the regulation of the growth inhibitory effects of AZD6244 in melanoma. We specifically focused these studies on Braf-mutant melanoma cells as these mutations are predominant in the common cutaneous melanomas, and ongoing clinical trials with AZD6244 require Braf mutation for patient enrollment. However, there is a clear need to determine if similar effects and combinations are important in other melanoma genotypes, including Nras-mutants and melanomas in which activating
kinase mutations are not detected. Further, although RPPA is a powerful technology that can be used to investigate both the expression and the activation states of proteins, we recognize that its scope is limited to investigations of targets for which validated antibodies are available. Whole-genomic approaches, such as mRNA or miRNA profiling, may suggest additional pathways that regulate sensitivity to AZD6244 in Braf-mutant melanomas. At the protein level, there remains a strong rationale to determine if the differential clinical outcomes seen with RAS-RAF-MAPK pathway inhibitors in vivo correlate with a threshold level of pathway inhibition. However, our functional testing of off-target pathways that are activated by, and correlate with resistance to, AZD6244 supports performing similar investigations with other agents. We believe this approach will identify combinatorial approaches with increased efficacy for this highly aggressive disease.

Disclosure of Potential Conflicts of Interest

P.D. Smith owns stock in AstraZeneca. The other authors disclosed no potential conflicts of interest.

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Figure 6. Continued. Identical experiments were performed in the MEL624 cell line for the effects on signaling proteins (C) and cell death (D).


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