Selective Growth Inhibition in BRAF Mutant Thyroid Cancer by the Mitogen-Activated Protein Kinase Kinase 1/2 Inhibitor AZD6244

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Context: Activating mutations in the BRAF gene, primarily at V600E, are associated with poorer outcomes in patients with papillary thyroid cancer. MAPK kinase (MEK), immediately downstream of BRAF, is a promising target for ras-raf-MEK-ERK pathway inhibition.

Objective: The objective of the investigation was to study the efficacy of a MEK1/2 inhibitor in thyroid cancer preclinical models with defined BRAF mutation status.

Experimental Design: After treatment with the potent MEK 1/2 inhibitor AZD6244, MEK inhibition and cell growth were examined in four BRAF mutant (V600E) and two BRAF wild-type thyroid cancer cell lines and in xenografts from a BRAF mutant cell line.

Results: AZD6244 potently inhibited MEK 1/2 activity in thyroid cancer cell lines regardless of BRAF mutation status, as evidenced by reduced ERK phosphorylation. Four BRAF mutant lines exhibited growth inhibition at low doses of the drug, with GI50 concentrations ranging from 14 to 50 nM, predominantly via a G1/S arrest, comparable with findings in a sensitive BRAF mutant melanoma cell line. In contrast, two BRAF wild-type lines were significantly less sensitive, with GI50 values greater than 200 nM. Nude mouse xenografts tumors derived from the BRAF mutant line ARO exhibited dose-dependent growth inhibition by AZD6244, with effective treatment at 10 mg/kg by oral gavage. This effect was primarily cytostatic and associated with marked inhibition of ERK phosphorylation.

Conclusion: AZD6244 inhibits the MEK-ERK pathway across a spectrum of thyroid cancer cells. MEK inhibition is cytostatic in papillary thyroid cancer and anaplastic thyroid cancer cells bearing a BRAF mutation and may have less impact on thyroid cancer cells lacking this mutation. (J Clin Endocrinol Metab 92: 4712–4718, 2007)

The majority of papillary thyroid cancer (PTC) cases exhibit molecular abnormalities that activate the ras-raf-MAPK kinase (MEK)/ERK signal transduction pathway (reviewed in Refs. 1, 2). These molecular lesions include activating mutations in the BRAF gene, activating RET gene rearrangements, RAS gene mutations, and methylation-induced silencing of the RASSF1A gene promoter. These abnormalities appear to be mutually exclusive in thyroid cancers (3–5), suggesting that any one of these events is sufficient to activate the pathway and confer a growth advantage on the thyroid cell harboring the lesion.

BRAF is activated by a V600E mutation in about 45% (29–83% in several studies) of cases of PTC and 24% of anaplastic thyroid cancer (ATC) (3). In studies of BRAF and PTC progression, a majority, including the largest multi-institutional series to date (6), associates a BRAF mutation with adverse clinical characteristics including extrathyroidal invasion, lymph node metastasis, and clinical recurrence. The molecular basis for the apparent virulence associated with BRAF mutations, compared with alternative molecular changes, is unclear.

In addition to thyroid cancer, BRAF-activating mutations are prevalent in melanoma (~59%), colorectal cancer (5–22%), serous ovarian cancer (~30%), and several other tumor types (7, 8). Solit et al. (9) reported that inhibition of MEK with CI-1040 efficiently inhibited tumor growth in BRAF mutant cell lines and xenografts, whereas ras mutant tumors were only partially inhibited. CI-1040 has an IC50 (half-maximal inhibitory concentration) for MEK1 of 17 nM, predominantly via a G1/S arrest, comparable with findings in a sensitive BRAF mutant melanoma cell line. In contrast, two BRAF wild-type lines were significantly less sensitive, with GI50 values greater than 200 nM. Nude mouse xenografts tumors derived from the BRAF mutant line ARO exhibited dose-dependent growth inhibition by AZD6244, with effective treatment at 10 mg/kg by oral gavage. This effect was primarily cytostatic and associated with marked inhibition of ERK phosphorylation. These data suggested a selective dependency on MEK activity in BRAF mutant tumors, offering a therapeutic rationale for MEK inhibition in the subset of tumors bearing this mutation (9).

Several MEK inhibitors have been studied in clinical trials to date. In addition to CI-1040, PD-0325901 and AZD6244 (ARRY-142886) are potent, specific inhibitors of MEK1 and MEK2. These drugs are noncompetitive with respect to ATP.
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and do not interact with the ATP-binding pocket; this may contribute to heightened specificity, compared with ATP-competitive kinase inhibitors. In a recent phase I/II clinical trial of PD-0325901, two of 27 patients with melanoma achieved a partial response, and eight patients achieved stable disease from 3 to 7 months (11). In posttreatment biopsies, phosphorylated (p) ERK levels were suppressed. AZD6244 has an IC<sub>50</sub> of 12 nM for MEK1 and MEK2 but greater than 10 μM for a panel of more than 40 other kinases (12). It inhibits growth of a variety of BRAF and K-ras mutant tumor cell lines at submicromolar concentrations. In (most) of these cell types, AZD6244 induces G<sub>0</sub>/G<sub>1</sub> cell cycle arrest, but in a few cell lines (Malme-3M and SK-MEL-2 melanoma cell lines), AZD6244 induces apoptosis. AZD6244 was generally well tolerated in a phase I trial at doses up to 100 mg twice a day. In a phase I study of AZD6244, stable disease longer than 5 months was seen in nine patients, with significant decrease in pERK after the dose in matched tumor samples (13). Phase II clinical trials of AZD6244 are currently ongoing in patients with melanoma, non-small cell lung cancer, colorectal cancer, and pancreatic cancer.

In the current study, we investigated the efficacy of AZD6244 in a panel of PTC and ATC cell lines and xenografts. We particularly focused on whether BRAF mutation status influenced response to this MEK1/2 inhibitor. Our data show that AZD6244 is effective in inhibiting thyroid cancer growth in a manner reflecting BRAF mutation status.

Materials and Methods

Cell lines

ARO and NPA were obtained from Dr. Guy Juillard (University of California, Los Angeles, Los Angeles, CA); KAT10, KAT18, and KAK1 from Dr. Kenneth Ain (University of Kentucky, Lexington, KY). Culture conditions were as follows: ARO-DMEM with 5% fetal bovine serum (FBS); KAT10 and KAK1-RPMI 1640 with 10% calf serum, 1 mm pyruvate, 0.1 mm nonessential amino acids, 1% penicillin-streptomycin; NPA-RPMI 1640, 10% FBS, 0.15% NaHCO<sub>3</sub>, 0.7 ng/ml gentamicin, 1 ml penicillin-streptomycin; H9262, 10% FBS, 0.15% NaHCO<sub>3</sub>, 0.7 ng/ml gentamicin. RPMI 1640 and 10% FBS.

MEK inhibitor treatment

U0126 (Sigma-Aldrich, St. Louis, MO) was prepared as a 10-mM stock solution in dimethylsulfoxide (DMSO). AZD6244 (AstraZeneca, Wilmington, DE) was prepared as a 1.6-mM stock solution in DMSO, following the manufacturer’s instructions. For analysis of MEK-ERK pathway inhibition, cultured cells were treated with indicated doses of inhibitors for 4 h. For growth analyses, cells were treated daily with indicated doses suspended in fresh media.

Growth analyses

Growth curves were performed in triplicate using the 3,4,5-dimeth-ylthiazol-2,5-diphenyltetrazolium (MTT) assay (M2128; Sigma-Aldrich) following the manufacturer’s instructions. Cells were seeded in 24-well plates using phenol red-free media. Media were changed daily in all experiments. To generate dose-response curves for each cell line, MTT absorbance was determined 5 d after exposure to AZD6244 or DMSO alone. This was used to calculate GI<sub>50</sub> by the method of Chou and Talalay (15), in which GI<sub>50</sub> is defined as the dose resulting in 50% inhibition of the MTT absorbance increase measured in control cells. In time-response curves, cells were treated with 400 nM AZD6244 or equal volume of DMSO with MTT assays performed daily for 6 d. Data are represented as the mean absorbance ± SEM, based on three to six independent incubations.

Fluorescence-activated cell sorter (FACS) cell cycle analyses

Thyroid cancer cells were treated for 48 h with 400 nM AZD6244 or vehicle control and then trypsinized and lysed in Hoechst 33258 staining solution for FACS analysis (0.56% Nonidet P-40, 3.7% formaldehyde, and 11 μg/ml Hoechst 33258 in PBS). Nuclei were analyzed using an LSR flow cytometer (BD Biosciences, Franklin Lakes, NJ) gated for single nuclei. The cell cycle profile was determined using 10,000 gated nuclei with ModFit LT 2.0 software (Verity, Topsham, MA).

Western blotting

Cells were treated for 4 h as described above and then washed with PBS and harvested by scraping with 1% sodium dodecyl sulfate lysis buffer [2% sodium dodecyl sulfate and 62.5 mM Tris (pH 6.8)]. Lysates were electrophoresed on 4–20% gradient polyacrylamide gels and transferred onto polyvinyl difluoride membranes. Blots were probed at 4°C overnight with primary antibody to pERK (CST, Beverly, MA; no. 9101) 1:1000 in 5% milk, pAKT (ser473, CST no. 9273), total AKT (CST no. 9272), phosphorylated c-Jun N-terminal kinase (INK) (Thr183/Tyr185, CST no. 9251), total JNK (CST no. 9252), p-p38Thr180/Tyr182, CST no. 9211), or total p38 (CST no. 9212). Anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:2000. Blots were visualized using Supersignal Pico Chemiluminescence (Pierce Chemical Co., Rockford, IL).

BRAF mutation analysis

Presence or absence of a BRAF V600E mutation was verified by automated sequencing at the Johns Hopkins DNA core facility.

Animal studies

Animal studies were approved by the Johns Hopkins Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines. ARO cells suspended in Matrigel (5 x 10<sup>5</sup> cells per 200 μl) were inoculated sc into the right flank of 4- to 6-wk-old female athymic nude (nu/nu) mice (Harlan Laboratories, Indianapolis, IN). Once palpable, tumors were measured at indicated intervals using Vernier calipers. Tumor volumes were calculated using the formula: tumor volume = length x width x height. (0.5236). After approximately 2 wk, tumors reached 0.1 cc in average size, and animals were sorted into groups of 10 to achieve equal distribution of tumor size in all treatment groups. Animals were untreated or treated twice daily, 5 d per week, with DMSO control, low-dose (10 mg/kg), medium-dose (30 mg/kg), or high-dose (100 mg/kg) AZD6244 administered orally by gavage tube. At the end of the experiment, animals were euthanized to CO<sub>2</sub> asphyxiation. Statistical analysis of differences in tumor volumes was performed using a two-tailed Student’s t test. Kaplan-Meier analysis was performed with statistical software (GraphPad Inc., San Diego, CA), using a 4-fold increase in tumor volume from onset of treatment as a threshold for tumor progression.

Immunohistochemistry

Two hours after treatment with AZD6244 100 mg/kg or DMSO control, mice were killed and tumors harvested in 10% paraformaldehyde overnight, followed by incubation in 70% ethanol. Sections were incubated with anti-pERK (1:250) using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine as chromogen and a hematoxylin counterstain.

Results

MEK inhibition

To determine the sensitivity of thyroid cancer cell lines to MEK inhibition, we initially treated ARO cells with AZD6244, and analyzed MEK inhibition by Western blotting for pERK (Fig. 1A). In ARO cells, there was almost complete...
MEK inhibition at an AZD6244 dose of 25 nM and an IC<sub>50</sub> of approximately 10 nM, consistent with earlier reports (12).

To examine the sensitivity in other thyroid cancer cell lines of MEK to AZD6244 inhibition, six thyroid cancer cell lines, and the colorectal cancer cell line HT29 as a positive control (12), were treated for 4 h with 200 nM AZD6244, the positive control MEK inhibitor U0126 (10<sup>−6</sup>/H9262/H9012), or a DMSO vehicle control (C). Evaluation for potential cross-reactivity with other ras-dependent signaling pathways. Thyroid cancer lines from B treated 5 h with 400 nM AZD6244 (A) or control (C). Active and total AKT, active and total JNK, and active and total p38 are shown.

**TABLE 1.** BRAF mutation status and 50% growth inhibitory concentrations of AZD6244

<table>
<thead>
<tr>
<th>BRAF status</th>
<th>GI&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<tr>
<td>KAK1 Mut</td>
<td>50</td>
</tr>
<tr>
<td>KAT10 Mut</td>
<td>42</td>
</tr>
<tr>
<td>NPA Mut</td>
<td>14</td>
</tr>
<tr>
<td>ARO Mut</td>
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</tr>
<tr>
<td>TPC1 WT</td>
<td>&gt;600</td>
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<tr>
<td>KAT18 WT</td>
<td>&gt;800</td>
</tr>
<tr>
<td>SKMel28 BRAF mut melanoma</td>
<td>23</td>
</tr>
<tr>
<td>BxPC-3 WT pancreatic</td>
<td>255</td>
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Mut, Mutated; wt, wild type.

levels of AKT activation, compared with the four BRAF mutant lines.

**Growth inhibition**

We next analyzed the dose-response pattern for inhibition of thyroid cancer cell proliferation by AZD6244. Table 1 illustrates the GI<sub>50</sub> for each cell line. Four lines bearing V600E BRAF mutations were all sensitive to AZD6244, with GI<sub>50</sub> values ranging from 14 to 50 nM. A positive control BRAF mutant melanoma line, SKMel28 (9), exhibited a similar GI<sub>50</sub> of 23 nM. In contrast, two BRAF wild-type thyroid cancer lines were somewhat resistant to AZD6244 with GI<sub>50</sub> values greater than 600 nM. TPC1 cells, bearing a Ret-PTC1 mutation that activates ras-raf-MEK-ERK pathway signaling, among other pathways (16), were resistant to the agent, as were KAT18 cells.

Examples of dose-response data are illustrated for the sensitive ARO line and the resistant KAT18 line (Fig. 2, A and C, respectively). Note that maximal growth inhibition for ARO cells was achieved with AZD6244 concentrations between 200 and 400 nM. In KAT18 cells, 50% growth inhibition was never achieved at levels up to 1600 nM.

In time-course analysis (Fig. 2, B and D), ARO cells were growth inhibited over a 6-d course, although we observed regrowth if the drug was removed. No significant growth inhibition over the time course was seen for KAT18 cells (data not shown). Cell cycle analyses are summarized in Table 2. In BRAF mutant thyroid cancer cell lines (KAK1, NPA, KAT10, ARO), AZD6244 resulted in a 35-55% reduction in S-phase nuclei. This change was accompanied by increases in the G<sub>S</sub>/G<sub>1</sub> fraction in KAK1, NPA, and ARO cells, whereas KAT10 cells exhibited a proportionally greater in-
crease in G2/M. The BRAF wild-type lines TPC1 and KAT18 exhibited no significant cell cycle changes. Figure 2E illustrates a representative FACS analysis in the BRAF mutant line KAK1. No increase in the sub-G0/G1 nuclear fraction, indicative of apoptosis, was seen in any of the tested cell lines.

Xenograft studies

Based on the encouraging in vitro data for MEK inhibition and growth arrest, we treated an ARO cell nu/nu mouse xenograft model of anaplastic thyroid cancer with three dose levels of AZD6244, corresponding to clinically achievable

<table>
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<tr>
<th></th>
<th>Control</th>
<th>400 nM AZD6244</th>
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<tr>
<td></td>
<td>G0/G1</td>
<td>S</td>
</tr>
<tr>
<td>KAK1</td>
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</tr>
<tr>
<td>TPC1</td>
<td>47.7</td>
<td>42.4</td>
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</table>

TABLE 2. Cell cycle distribution in thyroid cancer cells treated 48 h with MEK inhibitor AZD6244
levels in human phase I studies (13). In this model, ARO cells were allowed to grow to a mean tumor volume of 0.1 cc before initiation of treatment. Mice were treated 5 d of every 7. We observed growth inhibition at all doses tested, with a modest dose-dependent effect (Fig. 3A). Using Kaplan-Meier analysis for progression (Fig. 3B), the 100-mg/kg dose was highly significantly different from untreated control ($P < 0.005$). The 10- and 30-mg/kg doses were also significantly more effective than control ($P < 0.05$). We observed a moderate tendency of tumors to regrow during the 2-d intervals that the drug was withheld, consistent with a cytostatic, rather than cytotoxic, mechanism.

To verify that MEK inhibition was occurring at the same time as growth inhibition, we performed immunohistochemistry for pERK1 and pERK2, using vehicle-treated mouse tumors as a positive control and absence of primary antibody as negative control for pERK reactivity. In vehicle-treated ARO xenograft tumors, we observed intense pERK immunoreactivity with predominantly nuclear localization (Fig. 3C). In tumors harvested 2 h after treatment with AZD6244 (100 mg/kg), we observed a substantial decline in pERK reactivity, roughly comparable with the negative control (lacking primary antibody).

**Discussion**

Advanced papillary and anaplastic thyroid cancers typically exhibit radioiodine resistance. For patients with these thyroid cancers that have escaped radioiodine sensitivity, investigational agents including angiogenesis inhibitors and other approaches are the main treatment options. Among specific therapeutic targets in PTC and ATC, BRAF and its downstream signaling pathway seem especially promising. A significant percentage of aggressive PTC tumors, including the majority of tall cell variant PTC, exhibit BRAF mutations (17). In addition, nearly 25% of ATC tumors have BRAF mutations. There are currently no effective treatments for this most aggressive form of thyroid cancer.

Several attempts to inhibit the consequences of BRAF mutation in thyroid cancer have focused on the kinase itself. BRAF as a potential therapeutic target in thyroid cancer was validated by the efficacy in vitro of RNA interference ap-

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**Fig. 3.** MEK inhibitor AZD6244 inhibits ARO xenograft tumor growth. A, Tumor growth curve. ARO ATC cells (bearing a BRAF V600E mutation) were implanted sc in **nu/nu** mice for 2 wk and then left untreated, treated with DMSO vehicle, or treated twice daily, 5 d/wk, with one of three indicated doses of AZD6244. Data shown are mean tumor volumes for 10 mice in each treatment group. B, Kaplan-Meier analysis. Time to progression is determined by a 4-fold increase in tumor volume, compared with pretreatment baseline. C, Inhibition of MEK signaling in xenograft tumors. Immunohistochemistry with anti-pERK performed on tumor samples obtained 2 h after dosing with AZD6244 100 mg/kg ($\times 400$). Negative control lacking the primary antibody is also shown.
proaches (18, 19). Sorafenib (BAY 43–9006) is a multifunctional kinase inhibitor with modest activity against mutant BRAF [IC_{50} 38 nm for recombinant protein but 800 nm in a cell-based assay (20)] as well as activity against vascular endothelial growth factor receptor-2 and other kinases. Sorafenib at doses greater than 1 μm in vitro was effective in inhibiting pERK and growth in BRAF mutant thyroid cancer cell lines (18). Although phase II results for sorafenib in differentiated thyroid cancer have not yet been published, sorafenib had little or no activity in melanoma patients in a phase II trial, including patients with BRAF V600E mutations (21). Two additional kinase inhibitors with activity against BRAF, AAL881 and LBT613, also have shown preclinical activity against PTC (22).

An alternative strategy to block the raf-MEK-ERK pathway in cancer has focused on MEK. MEK1, and MEK2, in studies to date and appear to be the most critical downstream mediators of V600E BRAF signaling (8). Whereas ras and ret signaling activate multiple downstream pathways including ERK and phosphatidylinositol 3-kinase, BRAF signals primarily through the canonical MEK-ERK pathway. Interestingly, mutant V600E BRAF activates MEK proteins both directly and indirectly via c-Raf-1 (23). Activation of c-Raf-1 also could potentially lead to signaling via other important c-Raf-1 targets, such as apoptosis signal-regulating kinase-1 and nuclear factor-κB, in an MEK-independent fashion (24, 25). Although mutations in MEK could theoretically mimic the BRAF mutant phenotype and cause resistance to MEK inhibitors, mutations of MEK1 and -2 have not been reported in human cancer (COSMIC database v.28, 2007). The eventual emergence of multiple mutant kinase forms has been a major cause of drug resistance in the history of imatinib treatment in chronic myelogenous leukemia and gastrointestinal stromal tumor (26). It remains to be seen whether the approach of targeting a key downstream effector of a mutant kinase could be associated with less resistance than targeting the mutant kinase itself.

Based on the encouraging preclinical results of Solit et al. (9) with the MEK inhibitor CI-1040, primarily in melanoma, we elected to study AZD6244 in thyroid cancer. Our findings are consistent with observations for potent MEK inhibitors in other tumor cell types. Most significantly, we observed that all tested thyroid cancer lines bearing BRAF mutations were growth-arrested by AZD6244 but that BRAF wild-type thyroid cancer cell lines could be resistant to the drug, despite comparable inhibition of ERK phosphorylation. Because a majority of differentiated thyroid cancer tumor cells are believed to have up-regulated signaling through the ras-raf-MEK-ERK pathway, we had anticipated that partial sensitivity might be seen in some BRAF wild-type lines, analogous to the partial growth inhibition that we and others observed for ras mutation-bearing tumor cells. It seems likely that many BRAF wild-type thyroid cancer cell lines primarily use other signaling pathways to stimulate growth and can compensate for effective inhibition of MEK and ERK. In the case of a RET-PTC mutation such as in TPC1 cells, phosphatidylinositol 3-kinase, Rac, p38 MAPK, JNK, and other effector pathways are potentially activated by the mutant ret gene (16). We observed a significant level of AKT activation in TPC1 cells, which was unaffected by MEK inhibition.

AZD6244 is markedly more potent than U0126, a MEK inhibitor previously tested in preclinical thyroid cancer models. Prior studies using U0126 indicated an GI_{50} for growth inhibition greater than 10 μm in ARO cells and 8 μm in NPA cells (27). Both of these lines are highly sensitive to AZD6244. Members of our group recently showed that KAT10 cells had amplification of the PIK3CA gene (28). Despite this activation of a parallel growth-signaling pathway, KAT10 cells remained highly sensitive to AZD6244. We observed no enhancement of pAKT after treatment with the MEK inhibitor.

Our preclinical data indicate that an orally available MEK inhibitor can specifically and potently inhibit BRAF mutant thyroid cancer cells in vitro and in a xenograft model. The inhibitory effect that we observed was predominantly cytoplastic, rather than apoptotic, and was reversed when the drug was withdrawn. These results support the selection of AZD6244 for phase II clinical trials in PTC and ATC. It will be critical to test in a patient population whether response to this class of agents is predicted by BRAF mutation status, as our studies would indicate. A second implication of this preclinical study is that dosing to achieve constant target coverage may be required for maximal therapeutic effect.

Whereas it is tempting to invoke the concept of oncogene addiction (29) in interpreting the apparently selective efficacy of MEK inhibitors for BRAF mutant tumors, it is important to consider that resistance could emerge relatively promptly with chronic single-agent cytostatic therapy. MEK inhibitors have been shown to sensitize cancer cells to a number of other agents including radiation (30, 31). AZD6244 has been shown to enhance the efficacy of cytotoxic chemotherapy agents in preclinical colon cancer studies (12). A key challenge in thyroid cancer systemic therapy will be the identification of effective combinations, potentially combining targeted inhibition of BRAF signaling with other targeted or cytotoxic agents.

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