$BRAF^{V600E}$ Mutation Is Associated with Preferential Sensitivity to Mitogen-Activated Protein Kinase Kinase Inhibition in Thyroid Cancer Cell Lines

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Context: Mutually exclusive mutations of *RET*, *RAS*, or *BRAF* are present in about 70% of papillary thyroid carcinomas, whereas only the latter two are seen in poorly differentiated and anaplastic cancers. Although the signal output common to these oncoproteins is ERK, a recent report showed that only *BRAF* mutations consistently predicted responsiveness to MAPK kinase (MEK) inhibitors.

Objectives: Here we investigated whether sensitivity to MEK inhibition was determined by oncogene status in 13 human thyroid cancer cell lines: four with *BRAF* mutations, four *RAS*, one *RET/PTC1*, and four wild type.

Results: Growth of *BRAF* (+) cells was inhibited by the MEK antagonist PD0325901 with an IC_{50} of less than 5 nM. By contrast, *RAS*, *RET/PTC1*, or wild-type cells had IC_{50} of 4 nM to greater than 1000nM. Sensitivity was not predicted by coexisting mutations in *PIK3CA* or by *PTEN* status. Similar effects were obtained with the MEK inhibitor AZD6244. PD0325901 induced a sustained G1/S arrest in BRAF (+) but not *BRAF* (−) lines. PD0325901 was equipotent at inhibiting pERK1/2 after 2 h, regardless of genetic background, but pERK rebounded at 24 h in most lines. MEK inhibitor resistance was associated with partial refractoriness of pERK to further inhibition by the compounds. AZD6244 was more potent at inhibiting growth of NPA (*BRAF* +) than Cal62 (*KRAS* +) xenografts.

Conclusion: Thyroid cancers with *BRAF* mutation are preferentially sensitive to MEK inhibitors, whereas tumors with other MEK-ERK effector pathway gene mutations have variable responses, either because they are only partially dependent on ERK and/or because feedback responses elicit partial refractoriness to MEK inhibition. **(*J Clin Endocrinol Metab* 93: 2194–2201, 2008)**

Papillary thyroid carcinomas (PTC) are the most frequent type of thyroid malignancy. These tumors are associated with characteristic genetic alterations, which are believed to be involved in tumor initiation. These include rearrangements of the tyrosine kinase receptor oncogenes *RET* (1) or *NTRK1* (2), leading to illegitimate expression of the chimeric proteins RET/PTC (of which there are multiple variants) or TRK, respectively, and constitutive activation of their tyrosine kinase activities. Activating *RAS* mutations, particularly *NRAS* and *HRAS*, are seen in follicular thyroid carcinoma as well as follicular variant of PTC (3, 4). Activating mutations of *BRAF* are the most common genetic alterations in PTC (5–7). Moreover, *BRAF* mutations are also found in poorly differentiated or anaplastic thyroid carcinoma (8). The *BRAF* mutation is almost exclusively a thymine-to-adenine transversion at position 1799, leading to a valine-to-glutamate substitution at residue 600 (V600E) (9). Altogether, approximately 70% of PTCs harbor a mutation in either *RET*, *NTRK1*, *NRAS*, *HRAS*, or *BRAF*. These mutations are non-overlapping, suggesting that no selective advantage is derived by acquiring more than one of these abnormalities during tumor development (5, 7, 10). All these oncoproteins have the common property of activating MAPK kinase (MEK) and ERK pathway,

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Abbreviations: MEK, MAPK kinase; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PTC, papillary thyroid carcinoma.

leading to the hypothesis that inappropriate signaling through this pathway is critical to tumor initiation and transformation, and presumably for tumor maintenance.

The premise that MEK-ERK activity is required for viability of cancer cells in which the pathway is activated by upstream receptor mutations has been tested in thyroid cells. RET/PTC-induced activation of MEK-ERK in rat thyroid cells is blocked by small interfering RNA-mediated knockdown of BRAF but not CRAF (11). Knockdown of BRAF or pharmacological MEK inhibition disrupts RET-induced expression of a large set of genes, including functional clusters likely required for cell cycle progression and tumor invasiveness (12, 13). This in turn suggests that RAF proteins, and in particular BRAF, may represent legitimate therapeutic targets for patients with papillary thyroid cancer. Accordingly, treatment of human thyroid cancer cell lines with the small-molecule RAF kinase inhibitors AAL881 or LBT617, isoquinolines with submicromolar IC_{50} activity on wild-type RAF proteins and mutant BRAF, was effective at inhibiting growth of human thyroid cancer cells with endogenous *RET/PTC* or *BRAF* mutations (14). Although these compounds inhibit RAF, they also have inhibitory activity on other kinases (*i.e.* abl and kinase insert domain-containing receptor), and it is therefore not possible, based on this study alone, to conclude that RAF is a valid therapeutic target for thyroid cancers, regardless of the oncogenic event responsible for activating MEK-ERK (14, 15).

Recently Solit *et al.* (16) demonstrated that *BRAF* mutation predicted sensitivity to MEK inhibition in a panel of human cancer cell lines of different lineages. They showed that pharmacological MEK inhibition potently impaired tumor growth in *BRAF* mutant xenografts, whereas *RAS* mutant tumors were only partially inhibited. This study used the NCI60 cell line panel, which does not include thyroid cancer cell lines. After this observation and while this study was in progress, two groups examined the effect of MEK inhibition on a small panel of human thyroid cancer cell lines (17, 18) and found that sensitivity to MEK inhibitors was confined to cells with BRAF mutation. It remains unclear whether these findings can be generalized and whether thyroid cancer cell lines with *RAS* mutations in particular are insensitive to MEK inhibition. Here we examined these questions in a larger panel of thyroid cancer cell lines to determine the genetic determinants of MEK dependency for growth, using two highly selective MEK inhibitors.

Materials and Methods

Cell lines

The human thyroid carcinoma cell lines NPA, ARO, 8505c, WRO, C643, Cal62, Hth74, Hth83, and Kat 18 were maintained in RPMI 1640 supplemented with 10% fetal calf serum. The human papillary thyroid cancer cell line TPC-1 was maintained in DMEM with 10% fetal calf serum. The human thyroid carcinoma cell lines ACT1, TTA1, and OCUT1 were maintained in DMEM supplemented with 5% fetal calf serum. All experiments were performed with the cells grown in their respective media unless specified otherwise. The cell lines were genotyped by Sequenom mass spectrometry for mutations of *BRAF*, all *RAS* genes, and for the 31 most common *PIK3CA* mutations. Presence of *RET/PTC* rearrangements was examined by RT-PCR as described (19). The entire coding region of PTEN was sequenced in the following cell lines: NPA, ARO, 8505c, WRO, OCUT-1, ACT1, and TPC-1. No mutations were found.

Reagents

The MEK inhibitors PD0325901 and AZD6244 (ARRY-142886) are allosteric ATP noncompetitive inhibitors of MEK and were provided by Judith Leopold (Pfizer, Groton, CT) and Paul Smith (AstraZeneca, Cheshire, UK), respectively. The IC_{50} of PD0325901 for isolated MEK is 1 nM. The IC_{50} of AZD6244 is 12 nM against isolated MEK, and inhibits ERK phosphorylation in a range of cultured tumor cells with an IC_{50} of approximately 10 nM. We performed additional experiments using AZD6244 because while this study was underway, clinical development of PD0325901 was discontinued, making it desirable to explore the action of a compound with a similar mechanism of action that was still in clinical trials. The following antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): ERK1 rabbit polyclonal (sc-94), pERK mouse monoclonal (sc-7383). The Rb (4H1) mouse monoclonal antibody (no. 9309), the pMEK1/2 rabbit polyclonal antibody (no. 9121), and the pRb (ser780) rabbit polyclonal antibody (no. 9307) were from Cell Signaling Technology (Beverly, MA). The antiphosphatidylinositol 3-kinase (PI3K; p85) rabbit polyclonal antibody (no. 06–195) was from Upstate Cell Signaling Solutions (Charlottesville, VA). Fetal bovine serum and penicillin-streptomycin-L-glutamine were purchased from Life Technologies, Inc. (Gaithersburg, MD). Propidium iodide (PI) was purchased from Sigma (St. Louis, MO).

Growth curves

Cells were plated in triplicate into 6-well plates at 6×10^4 cells/well, and treated with or without the indicated concentrations of PD0325901

TABLE 1. Genotype of thyroid cancer cell lines used in this study

Cell line	$BRAF^{V600E}$	$NRAS^{Q61K}$	$HRAS^{G12A/Q61R}$	$KRAS^{G12R}$	$PIK3CA^{H1047R}$	RET/PTC1
WRO	wt	wt	wt	wt	wt	wt
8505c	+/+	wt	wt	wt	wt	wt
ACT1	wt	$+/^-$	wt	wt	wt	wt
ARO	$+/^-$	wt	wt	wt	wt	wt
C643	wt	wt	$G12A^{+/-}$	wt	wt	wt
Cal62	wt	wt	wt	+/+	wt	wt
Hth-74	wt	wt	wt	wt	wt	wt
Hth-83	wt	wt	$Q61R^{+/-}$	wt	wt	wt
Kat18	wt	wt	wt	wt	wt	wt
NPA	+/+	wt	wt	wt	wt	wt
OCUT-1	$+/^-$	wt	wt	wt	+/+	wt
TPC1	wt	wt	wt	wt	wt	yes
TTA1	wt	wt	wt	wt	wt	wt

or AZD6244, with media changes every 2 d. Cells were collected by trypsinization and counted in a Vi-Cell series cell viability analyzer (Beckman Coulter, Inc., Fullerton, CA).

Cell cycle analyses

Cal62, WRO, OCUT1, ARO, and NPA were plated in triplicate into 60-mm dishes at 2×10^5 cells/well. The following day cells were incubated with fresh medium with or without 10 nM PD0325901. Cells were collected at 24 and 48 h and fixed in 70% ethanol at −20 C overnight. Fixed cells were centrifuged and washed once with PBS. Five hundred microliters of DNA staining solution (distilled water, Triton X-100, and sodium citrate) were added followed by PI staining solution (final concentration of 30 μg/ml) and ribonuclease A (final concentration of 20 μg/ml) and the proportion of cells in S, G_2/M, and G_1/G_0 determined by fluorescence-activated cell sorting analysis using a Cell Lab Quanta flow cytometer (Beckman Coulter), at an excitation range of 488 nm (argon laser) and 620 BP for PI.

Western blotting

Cells were harvested by trypsinization, washed once with cold PBS, and lysed in a buffer containing 20 mM Tris (pH 7.4), 135 mmol/liter NaCl, 2 mmol/liter EDTA, 1% Triton X-100, 25 mmol/liter β-glycerophosphate, 1 mmol/liter sodium orthovanadate, 1 mmol/liter sodium fluoride, 1 mmol/liter phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 10 μg/ml EA64 for 20 min. Lysates were repeatedly passed through a G27 needle, centrifuged, and protein concentration determined using the Micro BCA kit (Pierce, Rockford, IL). For xenografted tumors, about 50–100 mg fresh frozen tissue were homogenized in lysis buffer in a power homogenizer (PT3000; Polytron, Duluth, GA). The tissue lysates were centrifuged and supernatants collected. Western blots were performed on 35 μg protein separated by SDS-10% PAGE using the indicated antibodies, except for Rb and pRb where the Western blots were performed on 10 μg protein samples separated by SDS-7.5% PAGE.

Tumor xenografts

Female nude^{nu} athymic mice (Charles River Laboratory, Inc., Wilmington, MA) of approximately 4–6 wk of age were injected sc in the right flank with either 10^7 NPA or 10^7 Cal62 cells suspended in 20% fetal bovine serum. Treatment was initiated when tumor volume approached about 500 mm^3 as estimated by measuring length and width with calipers (width^2 × length/0.52). Tumor-bearing mice were randomized into two groups consisting of a control group (vehicle only) and a treatment group. Mice were weighed at the start of the treatment and every 3 d during the course of therapy. AZD6244 was dissolved in a mixture of 0.5% hydroxypropylmethyl cellulose (Sigma-Aldrich) and 0.1% polysorbate (Tween-80, Sigma-Aldrich) to a concentration of 20 mg/ml. Treatments were administered by oral gavage in a volume of approximately 100 μl using a sterile animal feeding needle. A dose of AZD6244 was 100 mg/kg twice daily. Animals were killed by CO_2 anesthesia 4 h after the last dose of AZD6244. Tumors were measured every 3 d with calipers. At the time the animals were killed, the tumors were dissected free of vessels, fibrous tissue, and surrounding dermis. Tumors were then weighed, cut longitudinally to provide a representative fragment for immunohistochemistry, and the remainder flash frozen in liquid N_2 for subsequent protein or RNA isolation. All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center.

Statistical analysis

All data are presented as the mean ± SD. Statistical significance of differences observed in tumor volumes of treated and untreated animals was determined using the Mann-Whitney test. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS software for Windows version 14 (SPSS, Inc., Chicago, IL).

Results

The genotype of the lines used in this study is shown in Table 1. Of 13 thyroid cancer cell lines, four had *BRAF* mutation, four had *RAS* mutations (one *NRAS*, two *HRAS*, and one *KRAS*), one

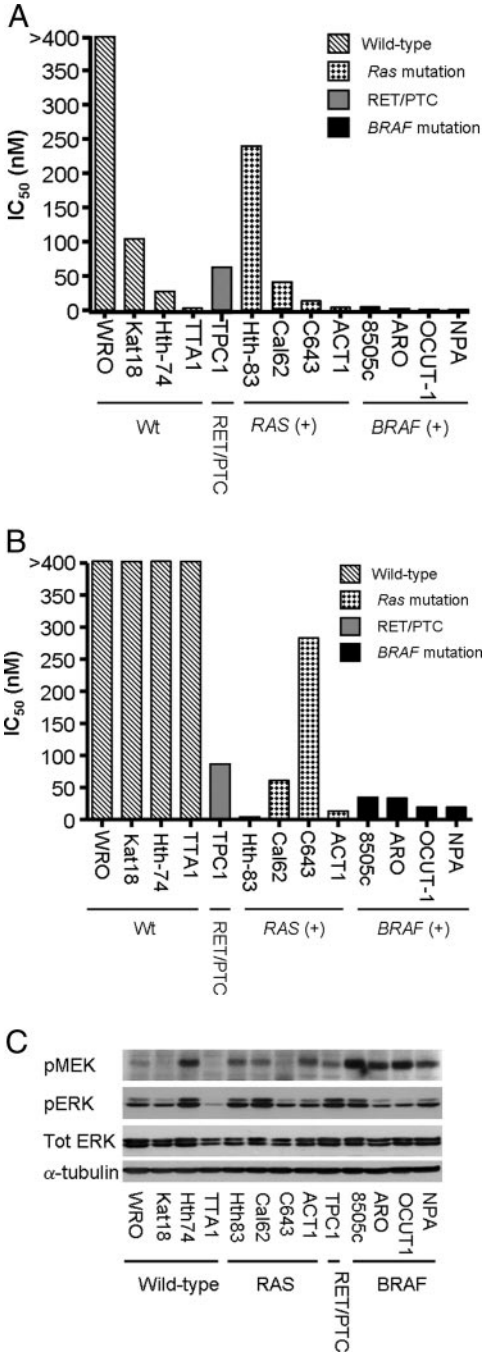

FIG. 1. A, Growth-inhibitory effects of PD0325901 on human thyroid cancer cell lines. The indicated thyroid cancer cell lines were treated for 6 d with PD0325901 (0.2–1000 nM), and the IC_{50} determined by nonlinear regression using GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA). B, Growth-inhibitory effects of AZD6244. The indicated cell lines were assessed for response to the MEK inhibitor AZD6244 as described in A, with concentrations ranging from 1 to 5000 nM. C, Basal level of pMEK and pERK in human thyroid cancer cell lines. Western blot of lysates of the indicated thyroid cancer cell lines extracted 72 h after incubation in serum-free media with 0.1% BSA. Membranes were incubated with the indicated antibodies.

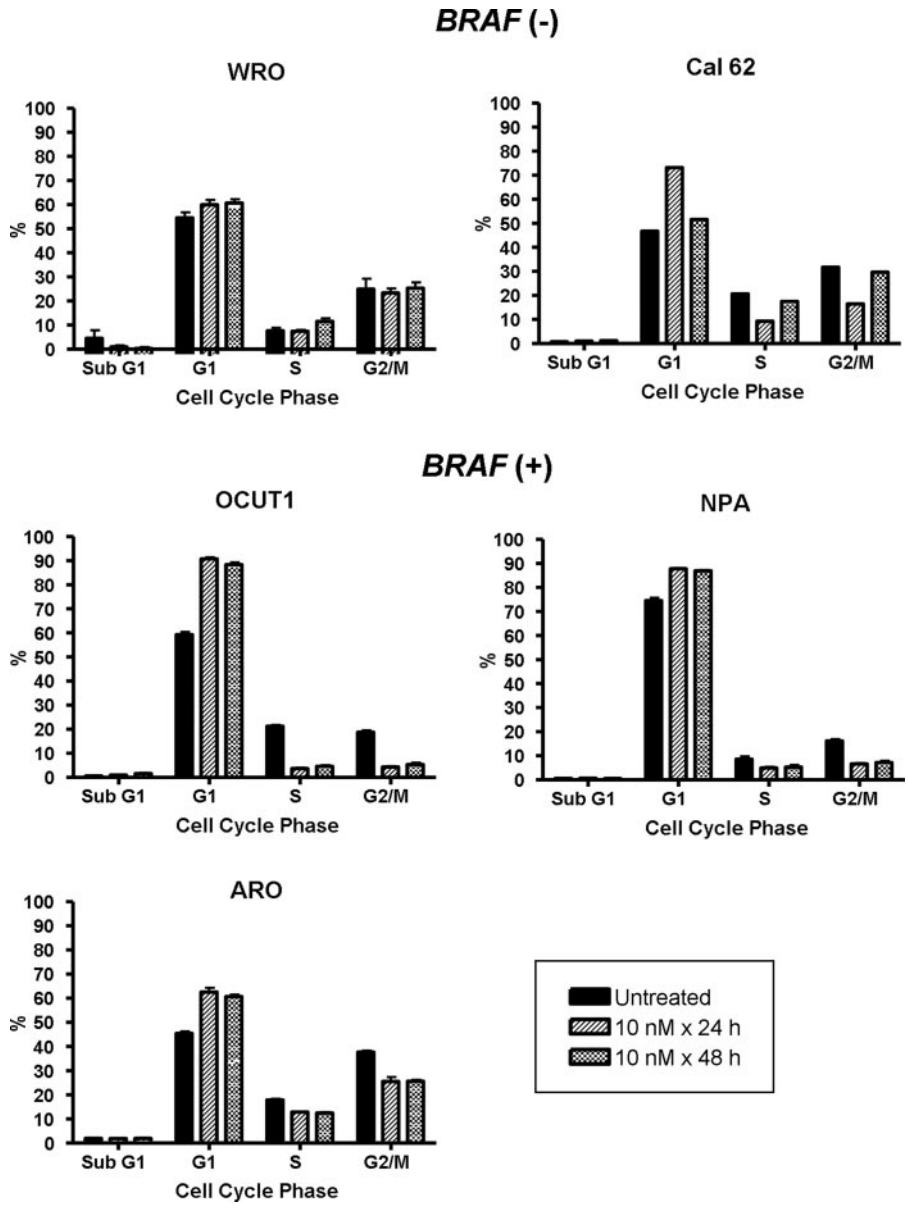

FIG. 2. Effect of PD0325901 on cell cycle progression of thyroid cancer cell lines with or without BRAF mutation. Fluorescence-activated cell sorting analysis of the indicated cell lines 24 and 48 h after treatment with 10 nM PD0325901. *Bars* indicate the percent of cells at the indicated stage of the cell cycle.

had a *RET/PTC1* rearrangement, and the remaining four were wild type for all genes tested (Table 1). Besides harboring a heterozygous $BRAF^{T1799A}$ substitution, the OCUT1 line was homozygous for the activating $PIK3CA^{H1047R}$ mutation.

Preferential inhibition of growth of BRAF (+) cell lines by PD0325901 and AZD6244

The effects of the MEK inhibitor PD0325901 and AZD6244 on growth of the 13 cell lines was determined by incubating them with a range of concentrations over a 6-d period. The four cell lines harboring a *BRAF* mutation (8505c, ARO, OCUT-1, and NPA) had IC_{50} less than 5 nM, whereas cells with *RAS* or *RET/PTC* mutations had more variable responses, with IC_{50} ranging from 5 to 250 nM, and WT cells had IC_{50} of 3–1000 nM when treated with PD0325901 (Fig. 1A). We also determined the IC_{50} of a different allosteric noncompetitive inhibitor of MEK, AZD6244, on the same panel of lines. As shown in Fig. 1B, all cell lines with BRAF mutation were highly sensitive to the compound, and the overall spectrum of activity closely resembled that of PD0325901. Some cell lines, such as Hth83, TTA1, and C643, exhibit slightly different responses. It is possible that these compounds may be metabolized differently, which may account for some of these changes. As shown in Fig. 1C, the basal levels of activation of MEK in serum-free conditions was highest in cell lines with *BRAF* mutation, whereas *RAS* and *RET/PTC* mutant lines tended to have an intermediate level of pMEK abundance. Overall, pMEK did not predict sensitivity to MEK inhibition. For example, TTA1 cells are very sensitive to MEK inhibition yet had undetectable pMEK, whereas Hth74 cells had high pMEK and were refractory to PD0325901. Basal pERK levels were highly variable, possible reflecting the fact that the MEK-ERK pathway is subject to multiple feedback regulatory controls.

PD0325901 induces a block in G_1 in BRAF (+) human thyroid cancer cells

Treatment of *BRAF* (+) cell lines (NPA, OCUT1, and ARO) with PD0325901 resulted in a cell cycle block in G_1 that was sustained through 48 h. There was no detectable sub-G1 peak, indicative of no induction of apoptosis. By contrast, the compound had no effect on cell cycle progression of WRO cells, which are wild type for these thyroid oncogenes. Cal62 cells, which harbor a homozygous *KRAS* mutation, exhibited a transient delay in G_1/S at 24 h, with a subsequent escape and progression to S and G_2/M after 48 h (Fig. 2). Treatment of the *BRAF* (+) cell lines NPA and OCUT1 with PD0325901 was associated with Rb hypophosphorylation, which was also accompanied by lower levels of total Rb. By contrast, Rb was largely unchanged or only slightly diminished in the *BRAF* (−) lines Kat 18 and WRO, respectively (Fig. 3).

Effect of PD0325901 on ERK phosphorylation in human thyroid cancer cell lines

pERK levels were markedly inhibited in all cell lines tested 2 h after treatment with PD0325901, regardless of genotype (Fig. 4A). There was a rebound in pERK after 24 h, which was more pronounced in cells that were comparatively less sensitive to the growth-inhibitory effects of the compound (*i.e.* WRO, Kat18, and TPC1), although there was also some recovery of ERK phosphorylation in ARO and TTA1 cells, which were exquisitely

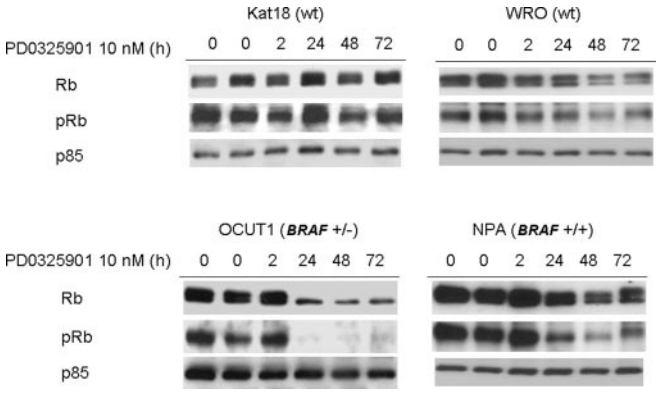

FIG. 3. Effect of PD0325901 on Rb phosphorylation in thyroid cancer cell lines with or without BRAF mutation. Western blots of cell lysates harvested from the indicated cell lines at various time points after addition of 10 nM PD0325901. Membranes were incubated with the indicated antibodies. Hybridization with p85 served as a loading control.

sensitive to MEK inhibitors (Fig. 4A). This property is distinct from effects of MEK inhibitors on other cancer cell lines, such as melanomas, in which PD0325901 induces a sustained inhibition of ERK phosphorylation through at least 72 h (not shown). We explored whether the rebound in ERK phosphorylation was associated with refractoriness to a rechallenge with MEK inhibitor.

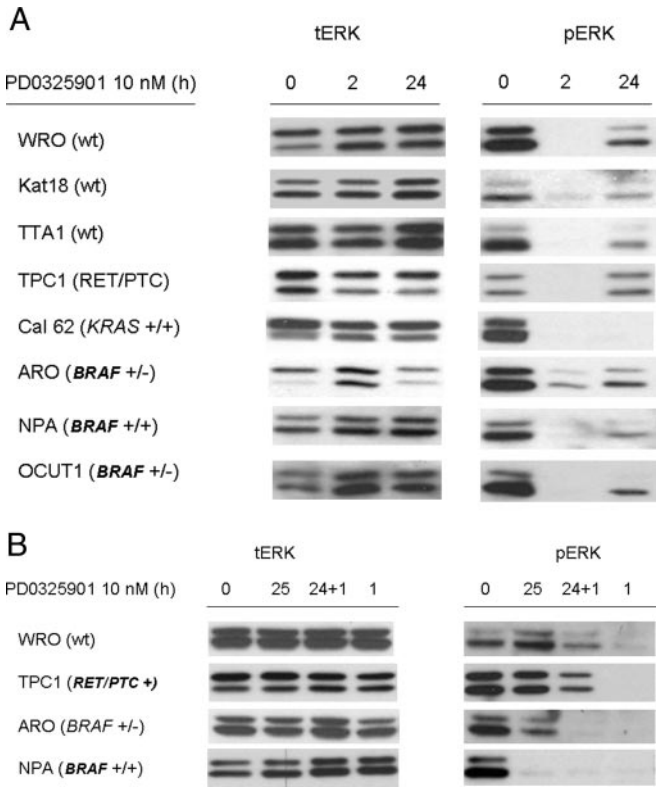

FIG. 4. A, Time course of ERK phosphorylation after treatment of thyroid cancer cell lines with the MEK inhibitor PD0325901. Cells were harvested at the indicated times after treatment with 10 nM PD0325901 and lysates Western blotted with antibody to total (*left panel*) or phosphorylated ERK (*right panel*). B, ERK phosphorylation rebound after 24 h of treatment with MEK inhibitor. Cells were harvested at the indicated times after treatment with 10 nM PD0325901 for 25 h by changing the media and the drug was readded or added for the first time 1 h before harvesting the cells. Lysates were Western blotted with antibody to total (*left panel*) or phosphorylated ERK (*right panel*).

For this we examined three cell lines showing the greatest increase in pERK 24 h after exposure to PD0325901, two of which were relatively resistant to the growth-inhibitory effects of the compound (WRO and TPC1) and one that was highly sensitive (ARO). In addition, we also tested NPA cells, which had no significant rebound in pERK. Cells were treated with PD0325901 for 24 h, and then refed with fresh compound for 1 h before analysis (Fig. 4B). TPC1 and WRO cells showed a near complete recovery of pERK after 25 h, and retreatment for 1 h failed to completely inhibit ERK phosphorylation, compared with the acute response seen after initial exposure to the compound. By contrast, pERK levels in ARO cells, which also recovered partially at 25 h, became undetectable after a rechallenge with the compound. NPA cells did not show any rebound in pERK after 25 h of exposure to the MEK inhibitor.

Effect of AZD6244 on NPA and Cal62 tumor xenografts

We compared the effects of MEK inhibition on xenografts of cells with high (NPA, $BRAF^{+/+}$) and intermediate (Cal62, $KRAS^{+/+}$) sensitivity to MEK inhibitors *in vitro*. It was not possible to examine the *in vivo* effects of AZD6244 on the more refractory lines (*i.e.* WRO, Kat18) because they did not grow as xenografts. NPA tumor xenografts grew by about 4-fold in vehicle-treated mice after 24 d. Growth of NPA was completely inhibited by AZD6244 (Fig. 5A). Growth suppression was associated with a decline in proliferation index in AZD6244, compared with vehicle-treated mice (8.8 ± 3.4 *vs.* 20.3 ± 5.6%, respectively; $P = 0.02$). Cal62 ($KRAS^{+/+}$) tumor xenografts grew by about 7-fold in the vehicle-treated mice after 24 d, and growth was blunted but not completely prevented by AZD6244 (Fig. 5B). Although the proliferation index was also lower in Cal62 xenografts in AZD6244-treated mice, compared with vehicle controls, the difference was not statistically significant (40 ± 11.5 *vs.* 20.3 ± 18.3%, vehicle *vs.* AZD6244, respectively; $P = 0.2$). There was an overall decrease in the level of pERK staining by immunohistochemistry in drug- *vs.* vehicle-treated controls in both tumor xenografts (data not shown).

Discussion

Activating mutations of *RET/PTC*, *RAS*, and *BRAF* are highly prevalent and do not overlap in papillary thyroid cancer specimens. RET/PTC and BRAF are involved in the early stages of thyroid tumorigenesis. The fact that they are mutually exclusive suggests that they share a common mechanism for transformation, thus implicating unregulated MEK-ERK signaling in thyroid cancer development (5, 7, 10). Alternatively, if any of these oncoproteins is activated by mutation, there may be no selective advantage for additional effector mutations in the pathway, which does not necessarily imply that ERK is the key driver of the process. A key question is whether thyroid cancers require continued MEK-ERK activity for their viability and whether tumor genotype determines this requirement. This information is of great consequence because MEK inhibitors are presently in clinical trials for several malignancies, including thyroid cancers. Solit *et al.* (16) reported that exquisite MEK dependency is a

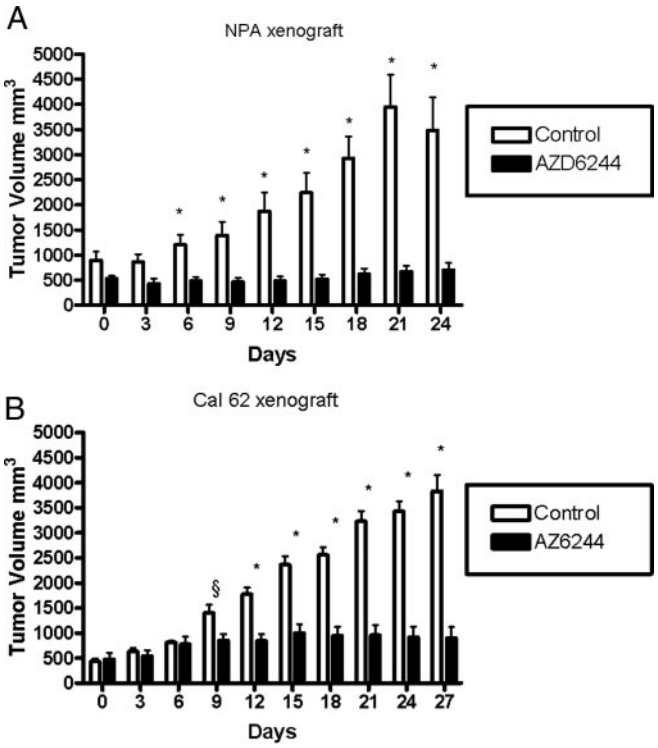

FIG. 5. Effect of treatment with AZD6244 on growth of thyroid cancer cell line xenografts in nude mice. A, NPA cells were implanted into the flanks of athymic *nu/nu* mice, and allowed to reach a volume of about 500 mm^3. Mice (n = 9 per group) were then treated with AZD6244 (100 mg/kg, twice a day) by gavage or vehicle. Data represent the mean ± SD of tumor volumes as measured with calipers in the two groups. *, $P < 0.02$ AZD6244 *vs.* vehicle-treated mice. At the time the animals were killed, tumor weights were 0.48 ± 0.25 *vs.* 0.09 ± 0.05, in AZD6244, respectively ($P = 0.04$). B, Cal62 xenografts were allowed to grow to about 500 mm^3 before treatment of mice (n = 4 per group) with vehicle or AZD6244 (100 mg/kg, twice a day) as described in Fig. 6A. Data represent the mean ± SD of tumor volumes as measured with calipers in the two groups. §, $P = 0.04$, *, $P < 0.02$ AZD6244 *vs.* vehicle-treated mice. At the time the animals were killed, tumor weight was 0.4 ± 0.09 *vs.* 0.09 ± 0.02 g, control *vs.* treatment, respectively ($P = 0.02$).

uniform property of cells with *BRAF* mutation, independent of cell lineage. Their data were particularly enriched with melanoma lines, and no thyroid cancer cells were studied. While this study was in progress, two reports showed that the MEK inhibitors AZD6244 (17) and CI-1040 (18) preferentially inhibited thyroid cancer cell lines with *BRAF* mutation. The data shown here reaffirm these observations, using a larger panel of lines that were verified to be genetically distinct from each other.

The predictive value of preclinical studies performed in human cancer cell lines is limited, in part because their growth requirements may change during adaptation to *in vitro* conditions. Findings are more likely to be generalizable if they are corroborated on multiple, independently derived lines, which was one of the objectives of this study. To this end we performed genomic fingerprinting on 28 human thyroid cancer cell lines obtained from different primary sources and found that many of them were not unique. The problem of cell line cross-contamination is well recognized in the literature. Estimates of the fraction of research papers whose conclusions may be compromised by the use of misidentified and cross-contaminated cell cultures approximate 15–20% (20). Based on our analysis, it is likely that most publications exploring the role of kinase inhibitors in thyroid cancer suffer from this problem. All but two of the 13 lines used in this study (WRO and Kat18) were examined by single-nucleotide polymorphism-comparative genomic hybridization or other single-nucleotide polymorphism-comparative genomic hybridization (SNP-CGH)-based genotyping approaches and verified to be distinct from each other.

Liu *et al.* (18) tested the response of a single cell line (C643) with a *RAS* mutation to the MEK inhibitor CI-1040 and found an IC_{50} comparable with that of cell lines with *BRAF* mutation. The four thyroid cancer cell lines with *RAS* mutations we examined varied markedly in their sensitivity to MEK inhibitors, consistent with the findings on the NCI60 lines harboring *RAS* mutations (16). None of the thyroid lines with *RAS* mutation had coexisting point mutations of *PIK3CA* or *AKT1* that could account for a primary dependence on this alternative pathway for growth. However, it is clear from recent cancer genome resequencing studies of colorectal and breast cancers that there are numerous other somatic mutations of genes encoding effectors that signal via PI3K (21). The basal level of pMEK in serum-free conditions was not predictive of the response of cell lines to MEK inhibitors.

Treatment of thyroid cell lines with *BRAF* mutations with MEK inhibitors was associated with Rb hypophosphorylation and impairment of progression into S and G_2/M. However, there was no accumulation of cells in a sub-G1 fraction, indicating no induction of apoptosis. Accordingly, the growth of NPA xenografts was completely inhibited by AZD6244, but there was no tumor regression or apoptosis. These data are consistent with the mode of action of MEK inhibitors in most melanoma cell lines with *BRAF* mutations, and on the effects of the pan-RAF inhibitors AAL881 and LBT613 in thyroid cancer cells (14). PD0325901 only induced a transient delay in G_1 in Cal62 cells, which harbor a homozygous *KRAS* mutation, and have intermediate sensitivity to the growth-inhibitory effects of the compound. This is consistent with a partial dependence on MEK signaling for growth, which was also apparent in the xenograft experiment. By contrast to these observations in cell lines and xenografts, pharmacological inhibition of MEK blocked lung tumor growth in mice with doxycycline-inducible expression of oncogenic *BRAF* or *KRAS* in alveolar epithelial cells (22). Moreover, CI-1040 induced apoptosis in these tumors, indicating that in this context MEK activity is required for *BRAF* or *RAS*-induced tumor cell viability in transgenic mice.

MEK inhibitors potently abrogated ERK phosphorylation at early time points in all cell lines tested. However, there was a significant recovery of pERK in most lines, irrespective of genotype or sensitivity to MEK inhibitors. This is quite distinct from melanoma cells with *BRAF* mutation, in which the inhibitory effects of PD0325901 on pERK are sustained. Readdition of the MEK inhibitor after 24 h in *BRAF* (+) ARO cells fully inhibited ERK phosphorylation, suggesting that the rebound might be attributable to efflux or rapid metabolism of the drug. The ATP-cassette binding transporters ABCB1 (Mdr-1; P-glycoprotein) and/or ABCG2 are expressed in thyroid cancer tissues and ARO cells (23), are known to interact with kinase inhibitors, and can mediate resistance to their action (24). Moreover, the Raf-MEK-

ERK pathway has been implicated in the regulation of Mdr-1 (25). By contrast, readdition of compound to WRO and TPC1 cells did not inhibit ERK phosphorylation to the same degree observed after initial exposure. The Ras/Raf/MEK/ERK pathway is subject to an extensive and complex set of feedback regulatory controls (reviewed in Ref. 26). Activated ERK phosphorylates Raf1 at multiple sites and appears to mediate its inactivation after mitogen stimulation (27), although the precise role of individual phosphorylation sites is controversial (28). ERK also phosphorylates BRaf at T753, which destabilizes Raf1-BRaf heterodimers (29). In addition to restoration of the stimulatory input into MEK, inhibition of Raf-MEK-ERK signaling results in a potent and durable down-regulation of expression of the dual-specificity MAPK phosphatases DUSP5 and DUSP6, which could also contribute to partial restoration of ERK activity (14). Constitutive activation of the PI3K pathway could also result in resistance to MEK-ERK pathway inhibitors at several levels. Thus, p21-activated kinase 1, whose activity is controlled in part by PI3K, phosphorylates and activates Raf1 as well as MEK (30). Moreover, the PI3K pathway plays a role in regulation of DUSP6 gene expression (31). Given the complexity of these interactions, we have not yet determined their possible role in the development of partial refractoriness of ERK to MEK inhibition in some of the cell lines with resistance to the growth-inhibitory effects of the compound. However, there is a sound conceptual basis that establishes the importance of duration of ERK activity on cell proliferation (32), emphasizing the potential significance of this issue in determining the efficacy of therapies targeting this pathway.

Thus, the $BRAF^{V600E}$ mutation predicts sensitivity to MEK inhibition in thyroid cancer cell lines. Some thyroid cell lines with *RAS* mutations are also highly sensitive, yet the factors that determine dependence on MEK activity are not understood. These findings provide rationale for testing MEK inhibitors in patients with radioiodine refractory and poorly differentiated thyroid cancers, particularly those with *BRAF* or *RAS* mutations. Clinical trials with these or similar compounds, presently ongoing, should determine whether tumor genotype will serve as a predictor of response to therapy. It is critical that potential mechanisms of failure to respond to MEK inhibitors in clinical trials be rigorously explored because the cumulative information derived from human thyroid cancer genetics, mouse models, and human cell lines strongly implicates this pathway in tumor initiation and maintenance.

Note Added in Proof

After this paper was accepted for publication, the original derivation of NPA and ARO cells has been questioned, with indications that they may not be of thyroid origin.

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