Inhibitors of Raf Kinase Activity Block Growth of Thyroid Cancer Cells with RET/PTC or BRAF Mutations In vitro and In vivo

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Abstract

Purpose: Papillary thyroid carcinomas are associated with nonoverlapping activating mutations of RET, NTRK, RAS and BRAF, which altogether are present in ~70% of cases. We postulated that compounds that inhibit a distal effector in the mitogen-activated protein kinase (MAPK) pathway would inhibit growth and tumorigenicity of human thyroid cancer cell lines with mutations of RET or BRAF.

Experimental Design and Results: We first examined the effects of AAL-881 and LBT-613, two inhibitors of RAF kinase activity, on RAF-MAPK/extracellular signal–regulated kinase (ERK) kinase (MEK)–ERK activation in thyroid PCCL3 cells after conditional induction of expression of H-RASG12V or BRAFV600E. Both compounds blocked RAS and RAF-dependent MEK and ERK phosphorylation. They also potently blocked MEK phosphorylation in human thyroid cancer cell lines with either RET/PTC1 (TPC1) or BRAFV600E (NPA, ARO, and FRO) mutations. Inhibition of ERK phosphorylation was transient in TPC1 and ARO cells, with recovery of ERK phosphorylation associated with concomitant down-regulation of the MAPK phosphatases MKP-3 and DUSP5. Both compounds inhibited growth of all cell lines, with LBT-613 being ~10-fold more potent than AAL-881. TPC1 cells were more sensitive to growth inhibition (IC50 0.1-0.25 and ~0.05 μmol/L for AAL-881 and LBT-613, respectively) than BRAF (+) lines (IC50 2.5-5 and 0.1-0.5 μmol/L, respectively). Growth inhibition was associated with G1 arrest, and induction of cell death. Growth of ARO and NPA tumor xenografts was inhibited by LBT-613 or AAL-881. MEK and ERK phosphorylation was inhibited by both compounds in ARO but not in NPA cell xenografts.

Conclusions: Compounds that inhibit kinase activity are effective growth inhibitors for poorly differentiated thyroid cancer cell lines with either RET or RAF mutations, and hold promise for treatment of most forms of papillary thyroid carcinoma.

Papillary thyroid cancers are the most common type of thyroid malignancy. They are associated with characteristic genetic defects. Notable among them are the tyrosine kinase receptor oncogenes RET/PTC and NTRK, which arise by intrachromosomal inversions or translocations leading to expression of chimeric proteins, and constitutive activation of their respective tyrosine kinases (1). Recently, an activating mutation of BRAF was found to be the most common oncogene thus far identified in sporadic forms of the disease (2). The mutation was almost exclusively a thymine to adenine transversion at position 1799, previously designated as 1796, leading to a valine-to-glutamate substitution at residue 600 (V600E; ref. 3). The initial observations have now been confirmed by other reports finding a prevalence of this mutation in 29 to 83% of papillary thyroid carcinomas (PTC; reviewed in ref. 4). Overall, of the 916 PTC reported to date, 42% were positive for the BRAFV1799A mutation. In addition, activating mutations of the three RAS genes are also found in a smaller subset of PTCs (5–7), particularly follicular variant forms of these cancers (8).

Several groups have examined PTCs for the presence of RET/PTC, BRAF and RAS mutations, all of which can activate the mitogen-activated protein kinase (MAPK) signaling pathway (2, 9, 10). Altogether, 177 PTCs were studied, and one of these alterations was present in ~70% of tumors. However, there was no single PTC with a mutation of more than one of these genes. This absence of overlap provides compelling genetic evidence for a requirement of mutation of MAPK signaling components for transformation to PTC. This is consistent with evidence that RET/PTC–induced dedifferentiation (11) and thyroid-stimulating hormone–independent growth (12) require activation of the MAPK pathway in thyroid cell lines. Several lines of evidence indicate that the deregulated kinase activity in PTC occurs early in tumorigenesis and may be important for tumor maintenance (reviewed in ref. 13).
Targeted inactivation of protein kinases involved in cancer pathogenesis is an attractive strategy for therapy that has already shown notable successes. This objective is often achieved through small-molecule inhibitors that compete with ATP for binding to the deregulated kinase. Several compounds have been found to inhibit RET kinase activity, and as such may be useful in treatment of patients with medullary thyroid carcinomas associated with activating RET point mutations, or PTC with RET/PTC rearrangements (14–16). However, RET/PTC rearrangements are present in a small fraction of sporadic PTC from adults. Moreover, as outlined above, PTCs are associated with nonoverlapping mutations of other genes signaling along the MAPK pathway. Indeed, selective knockdown of BRAF (but not CRAF) abrogates RET/PTC3-induced extracellular signal–regulated kinase (ERK) phosphorylation in thyroid cells (17). These data suggest that blocking RAF activity may be a logical site to interfere with the effects of the RET/PTC, RAS and RAF oncproteins in these cancers.

Therapeutic interest in RAF inhibitors has been heightened because RAF kinase activity is required for the transforming effects of several oncogenes. Thus, RAF binds to and is a direct effector for RAS, and is required for the transforming effects of RAS oncogenes (18). Moreover, RAF kinase is activated in cells with increased growth factor signaling, such as that seen in tumors with activating mutations of the epidermal growth factor receptor (EGFR) gene family. As mentioned, this is also the case in thyroid cancer, where RET and NTRK are the paradigmatic constitutively active receptors associated with RAF activation.

Targeted compounds designed against RAF that have entered clinical trials include antisense oligonucleotides against human CRAF, such as ISIS 5132 and LerafAON (reviewed in ref. 19), and small-molecule RAF inhibitors. Among the latter, BAY 43-9006 is a bis-aryl urea that inhibits wild-type and BRAFV600E kinase activity in vitro that is presently in phase 2 clinical trials for various cancer types. Although this effect requires micromolar concentrations of the inhibitor, these are within the plasma concentrations achieved in vivo. As this compound also inhibits KDR, platelet-derived growth factor receptor, and KIT, it is still not clear whether the favorable results observed thus far in several cancer types is due primarily to RAF kinase inhibition. Here we examine the effects of two potent inhibitors of RAF kinase activity on signaling and growth of thyroid cancer cells harboring endogenous activating mutations of either RET or BRAF. We report that inhibition of RAF kinase activity is effective in blocking growth of all lines at submicromolar concentrations, supporting the concept that distal inhibitors of the MAPK pathway may be promising therapeutic agents for most cases of advanced papillary thyroid cancer.

**Materials and Methods**

**Cell lines.** The human thyroid carcinoma cell lines NPA, ARO and FRO were maintained in RPMI 1640 supplemented with 10% FCS. The human papillary thyroid cancer cell line TPC-1 was maintained in DMEM with 10% FCS. PCCL3-Ras25, PCCL3-Raf9-6 and PCCL3-RET/PTC3 are derived from the well-differentiated, nontransformed rat thyroid cell line PCCL3 that conditionally express H-RASG12V, BRAFV600E or RET/PTC3, respectively, in a doxycycline-dependent manner (20–22). The PCCL3 derived cell lines were propagated in H4 complete medium, which consisted of Coon’s modification of Ham’s F12 media containing 5% FCS, glutamine (286 μg/mL), apotransferrin (5 μg/mL), hydrocortisone (10 μmol/mL), insulin (10 μg/mL), thyroid-stimulating hormone (10 mIU/mL), penicillin, and streptomycin.

**Reagents.** The RAF inhibitors NVP-AAL881-NX (hereafter referred to as AAL881) and NVP-LBT613-AG-8 (LBT613) are isoquinoline compounds (Novartis, Cambridge, MA). The entire spectrum of in vitro activity of these compounds on various kinases will be reported elsewhere. Briefly, the IC₅₀ (in micromolar) of AAL881 and LBT613 on BRAFV600E was 0.22 and 0.21; on wild-type BRAF, 0.94 and 0.2; on CRAF, 0.43 and 0.12; on C-ABL, 0.96 and 0.11; and on KDR 0.25 and 0.03, respectively. LBT613 was active against RET (0.55 μmol/L) whereas AAL881 had very low activity (5.8 μmol/L). The compounds had low or no activity (IC₅₀ > 1 μmol/L) on human epidermal growth factor receptor 1, human epidermal growth factor receptor 2, platelet-derived growth factor receptor β, protein kinase A, protein kinase B, phosphoinositide-dependent protein kinase 1, insulin-like growth factor-I receptor, or the insulin receptor. The phosphorylated MAPK/ERK kinase (pMEK, Ser217/221) rabbit polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): ERK1 rabbit polyclonal, pERK mouse monoclonal, MEK1 rabbit polyclonal, and MEK2 rabbit polyclonal. Propidium iodide, thyroid-stimulating hormone, insulin, apotransferrin, and hydrocortisone were purchased from Sigma (St. Louis, MO). The MEK inhibitor PD98059 was purchased from Calbiochem (San Diego, CA). Coon’s modification of Ham’s F12 media was purchased from Irvine Scientific (Irvine, CA). Fetal bovine serum and penicillin-streptomycin-1-glutamine were purchased from Life Technologies, Inc. (Gaithersburg, MD).

**Quantitative reverse transcription-PCR for MKP-3 and DUSP5 mRNA.** The effects of the RAF inhibitors on expression of the cytoplasmic (MKP-3) and nuclear (DUSP5) selective ERK phosphatases was measured by quantitative reverse transcription-PCR as described (23) using the following primers: MKP-3, 5'-ggcagattgagcttctgc-3' and 5'-tccctttccctgacaca-3'; DUSP5, 5'-ctacagggcatgattgca-3' and 5'-cttgccc-tctttttcttgacaca-3'.

**Fig. 1.** AAL881 and LBT613 inhibit MEK and ERK phosphorylation induced by oncogenic HRAS or BRAF in PCCL3 cells. PC-Ras25 and PC-BRAFV600E-6 cells were incubated with or without 1 μg/mL doxycycline for 24 hours in the presence or absence of PD98059 (75 μmol/L), AAL881 (10 μmol/L), or LBT613 (10 μmol/L). Protein extracts were Western blotted with the indicated antibodies. The double arrows in the ERK lanes point to ERK1 and ERK2, respectively.
Growth curves. Cells were plated in triplicate into six-well plates at 4 × 10^4 (TPC-1) or 2 × 10^5 per well (NPA, ARO, and FRO) and treated with or without the indicated concentrations of AAL881 or LBT613, with media changes every 2 days. Cells were collected by trypsinization and counted in a Z1 Coulter particle counter (Beckman Coulter, Inc., Fullerton, CA).

Determination of cell death. TPC-1, NPA, ARO, and FRO were plated into six-well plates at 1 or 5 × 10^5 cells per well and treated with or without the indicated concentrations of AAL881 or LBT613. Every other day, detached cells were collected from the medium and counted. On the final day, detached cells were collected and attached cells were trypsinized and counted separately. Detached cells were confirmed to be dead by trypan dye exclusion. The percentage of dead cells was calculated by addition of all detached cells collected during the course of the experiment and dividing this by the total number of cells (i.e., sum of detached and attached cells).

Effects of Raf inhibitors on cell cycle. TPC-1, NPA, ARO, and FRO were plated into six-well plates at ~60% confluence. The following day, cells were incubated with fresh medium with or without 10 μmol/L AAL881 or LBT613. Cells were collected at 24 and 48 hours and fixed in 70% ethanol at 4°C overnight. Fixed cells were centrifuged and washed once with PBS. Five hundred microliters of propidium iodide staining solution (50 μg/mL propidium iodide, 50 μg/mL RNase A) were added and the proportion of cells in S, G2-M, and G1-G0 was determined by fluorescence-activated cell sorting (FACS) analysis using a Coulter EPICS XL flow cytometer (Miami, FL), at an excitation range of 488 nm (argon laser), and 620 BP for propidium iodide.

Western blotting. Cells were harvested by trypsinization, washed once with cold PBS, and lysed in a buffer containing 20 mmol/L Tris (pH 7.4), 135 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100, 25 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL pepstatin, 10 μg/mL aprotinin, and 10 μg/mL EA64 for 20 minutes. Lysates were repeatedly passed through a G27 needle, centrifuged, and protein concentration was determined using a Micro BCA kit (Pierce, Rockford, IL). About 50 to 100 mg of fresh frozen tumor xenografts were homogenized in lysis buffer in a power homogenizer (Polytron PT3000). The tissue lysates were centrifuged and supernatants were

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Fig. 2. Inhibition of phosphorylation of MEK and ERK in thyroid cancer cell lines by AAL881 and LBT613. A and B, cell lines were incubated with the indicated compound (10 μmol/L) for the stated time. Protein extracts were Western blotted with the indicated antibodies. C and D, quantitation of the Western blots shown in A and B. Columns, percent change compared with untreated cells in pMEK and pERK after normalization for total MEK and ERK, respectively.
Western blots were done on 30 to 60 μg of protein separated by SDS-10% PAGE using the indicated antibodies.

**Tumor xenografts.** Female nu/nu athymic mice (Harlan, Indianapolis, IN), 4 to 6 weeks of age, received s.c. injections in both shoulders of 1 × 10⁷ ARO or NPA cells suspended in 20% fetal bovine serum. Treatment was initiated when tumor volume approached 500 mm³ as estimated by measuring length, width, and depth with calipers. Tumor-bearing mice were randomized into groups of four to five before therapy. Mice were weighed at the start of the treatment and every 3 to 4 days during the course of the therapy. AAL881 and LBT613 were dissolved in 100% ethanol to a concentration of 100 mg/mL. For AAL881, the drug was further diluted to 10 mg/mL in 5% D-(+)-glucose/cremaphor EL (Sigma). For LBT613, the drug was diluted to 2.5 mg/mL. Vehicle treatment consisted of 100% ethanol diluted in 5% D-(+)-glucose/cremaphor. Treatments were administered by gavage in a volume of ~0.25 mL using a sterile animal feeding needle. Dose of AAL881 was 0.1 mg/g twice daily. For ARO xenografts, LBT613 was also 0.1 mg/g twice daily. Because of toxicity manifesting as excessive weight loss, LBT613 dose in mice with NPA cell xenografts was reduced from 0.05 to 0.025 mg/g twice daily. Animals were sacrificed by CO₂ anesthesia. At autopsy, tumors were measured with calipers and dissected free of vessels, fibrous tissue, and surrounding dermis.

**Fig. 3.** Rapid decrease in expression of the ERK dual-specificity phosphatases MKP-3 and DUSP5 in thyroid cancer cell lines by AAL881 and LBT613. A and B, quantitative reverse transcription-PCR of MKP-3 and DUSP5 mRNA in thyroid cancer cell lines treated with 10 μmol/L AAL881 or LBT613 for the indicated times.

**Fig. 4.** RAF inhibitors inhibit growth of thyroid cancer cell lines. TPC1, NPA, ARO, and FRO cells were incubated with the indicated concentration of either AAL881 (top) or LBT613 (bottom) for 6 days with medium change every 2 days. Columns, mean of cell counts of triplicate wells; bars, SD.
Tumors were then weighed, cut longitudinally to provide a representative fragment for immunohistochemistry, and the remainder was flash frozen in liquid N2 for subsequent protein or RNA isolation.

**Sequencing of BRAF cDNA.** BRAF cDNA was amplified by PCR from ARO and NPA cell cDNA using the following primers: forward (BRAF 173-191), 5'-gctgcggaccctgccattc-3'; reverse (BRAF 2254-2274), 5'-aaac-cagcccgattcaaggag-3'. Unidirectional sequencing was then done using overlapping primers covering the entire sequence (sequences available on request).

**Results**

AAL881 and LBT613 inhibit RET/PTC-, RAS-, and BRAF-induced ERK activation in rat PCCL3 cells. The activity of the kinase inhibitors was tested in thyroid PCCL3 cells previously modified to express RET/PTC3, H-RAS G12V, or BRAF V600E in a doxycycline-dependent manner. As expected, the MEK inhibitor PD98059 blocked stimulation of ERK phosphorylation by all the upstream effectors. The RAF inhibitors AAL881 and LBT613 decreased oncprotein-induced MEK and ERK phosphorylation after 2 hours. To achieve sustained inhibition through 24 hours, incubation with 10 \( \mu \text{M} \) AAL881 or LBT613 was required (Fig. 1).

Effects of AAL881 and LBT613 on MEK and ERK phosphorylation in human thyroid cancer cell lines. We next examined whether these compounds had activity on the MAPK pathway in human thyroid cancer cell lines known to harbor the following oncogenes: TPC1, RET/PTC1 (24); NPA, ARO, and FRO, BRAF\(^{V600E}\) (2, 25). Of note, NPA and ARO cells also have loss-of-function p53 mutations (26). Treatment of all cell lines except NPA with AAL881 resulted in inhibition of MEK and ERK phosphorylation by 1 hour (Fig. 2A). Whereas inhibition of MEK phosphorylation was sustained through 24 hours, inhibition of ERK phosphorylation was transient in TPC1 and ARO cells and had partially recovered by 6 and 24 hours. LBT613 had a more potent effect than AAL881, but the pattern of inhibition was comparable to that of AAL881 in all cell lines, consistent with a similar mode of action. Despite rapid, profound, and sustained inhibition of MEK phosphorylation in all cell lines by LBT613, ERK phosphorylation either declined with delayed kinetics (NPA or FRO) or partially recovered after an initial decrease (ARO). As opposed to AAL881, LBT613 blocked MEK activation and reduced ERK phosphorylation in NPA cells (Fig. 2B). The explanation for the rebound activation of ERK in the face of continued inhibition of MEK is not clear. One possibility is that RAF inhibition may decrease expression of ERK phosphatases, thus impairing ERK dephosphorylation. As shown in Fig. 3A and B, both AAL881 and LBT613 evoked a rapid and sustained decrease of mRNA abundance of the ERK-specific phosphatases MKP-3 and DUSP5. These data are consistent with the concept that decreased expression of ERK phosphatases resulting from RAF kinase inhibition may dampen the overall blockade of ERK activation.

AAL881 and LBT613 inhibit growth of human thyroid cancer cell lines and induce cell cycle block in G1. Treatment with AAL881 or LBT613 for 6 days caused a dose-dependent inhibition of growth of all human thyroid cancer cell lines (Fig. 4). LBT613 was \(~5\)-fold more potent in all lines tested. There was a \(~10\)-fold difference in IC\(_{50}\) between TPC1 cells (which harbor RET/PTC1) and NPA, ARO, or FRO cells (which harbor the BRAF\(^{V600E}\) mutation) for both inhibitors. Thus, IC\(_{50}\) for AAL881 and LBT613 in TPC1 cells was \(~0.1\) to \(~0.25 \mu \text{M}\).

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**Fig. 5.** RAF inhibitors arrest thyroid cancer cell lines in the G0-G1 phase of the cell cycle. The indicated cell lines were incubated for 48 hours with either 10 \( \mu \text{M} \) AAL881 or LBT613 and then harvested for determination of cell cycle distribution by fluorescence-activated cell sorting using standard protocols.
and 0.05 μmol/L, respectively. By contrast, IC50 in NPA, ARO, and FRO cells was 2.5 to 5 and 0.1 to 0.5 μmol/L, respectively. The greater potency of LBT613 on TPC1 cells could be due to the fact that besides working as a RAF inhibitor, this compound also inhibits RET kinase in vitro. However, the more potent effects of the RAF inhibitors on TPC1 cells cannot be solely due to a combined effect on both kinases because AAL881 has similar relative potency on TPC1 versus the BRAF mutant lines, yet has no effect on RET kinase activity in vitro.

Treatment with RAF inhibitors for 48 hours impaired progression into S and G2-M and caused G0-G1 arrest in all cell lines (Fig. 5). We did not observe a sub-G1 fraction in the FACS experiments, presumably because these were done after a 48h incubation with inhibitor, a time when few cells were detaching from the dishes. However, AAL881 and LBT613 did induce cell death in a concentration-dependent manner in TPC1 cells over a 6-day period (Fig. 6). The RAF inhibitors had a similar effect on NPA, ARO, and FRO cells but at much higher concentrations. These data point again to the higher sensitivity of TPC1 cells to RAF inhibition. However, growth inhibition in all cell lines is likely independent of effects on cell survival because concentrations that do not alter cell viability markedly inhibit growth (i.e., 5 μmol/L AAL881 and 0.5 μmol/L LBT613).

**AAL881 and LBT613 inhibit growth of tumor xenografts of ARO and NPA cells.** Athymic mice were treated with the indicated dose of inhibitors after ARO and NPA tumor xenografts were allowed to grow to ~500 mm3. Xenograft experiments were not done with TPC1 cells because these cells did not consistently form tumors in vivo and had a much slower growth rate. ARO xenografts grew by ~5-fold in vehicle-treated mice after 15 days and treatment with AAL881 and LBT613 markedly blunted this growth (Fig. 7A). Dosing with 0.1 mg/g LBT613 was associated with considerable toxicity requiring sacrifice of treated animals after 6 days. By contrast, AAL881 was well tolerated. NPA cell xenografts grew at a slower rate in vehicle-treated controls with a doubling time of ~3 weeks (Fig. 7B). A lower dose of LBT613 (0.025 mg/kg) was used in these animals, which resulted in stabilization of xenograft tumor size, with better tolerance. AAL881 was less effective although growth of xenografts was significantly diminished. Final ARO and NPA tumor weight was markedly lower than vehicle-treated mice (Fig. 7C and D).

We next examined the effect of RAF inhibitors on MEK and ERK phosphorylation in ARO cell tumor xenografts harvested ~3 hours after the final administration of the compounds. MEK and ERK1/2 phosphorylation were markedly decreased in xenografts from mice treated with either inhibitor (Fig. 8A and C). These data were confirmed by immunohistochemical staining with a pMEK antibody (not shown). By contrast, despite the fact that NPA tumor xenograft growth was entirely abrogated in mice treated with LBT613 and partially decreased by AAL881, MEK and ERK1/2 phosphorylation was not changed (Fig. 8B and D). The cause of the relative resistance to RAF inhibition by these compounds in NPA cells is not clear. The BRAF cDNA of NPA cells was sequenced and, in addition to the known activating mutation leading to the V600E substitution, a C-to-G change in nucleotide 356 was detected, resulting in a threonine-to-serine substitution at amino acid 119. This substitution was confirmed by sequencing in the opposite direction and was not present in ARO cells.

**Discussion**

The data presented in this article show that small-molecule inhibitors of RAF kinase effectively block growth of human thyroid cancer cell lines in vivo and in vitro. We believe all cell lines used in this study originally arose from PTCs. Whereas TPC1 and NPA cells are known to be from patients with this disease, the FRO and ARO lines were derived from undifferentiated or anaplastic thyroid carcinomas. These highly malignant tumors likely arise from pre-existing well-differentiated follicular or papillary carcinomas. Undifferentiated cancers arising from PTCs frequently have the **BRAF**V600E oncogene (25, 27), which is otherwise found exclusively in PTC. As both FRO and ARO lines harbor the **BRAF** mutation, it is likely that their lineage began as PTCs. As activation of MAPK signaling components through nonoverlapping somatic mutations is critical for thyroid cancer initiation, inhibition of distal steps in this pathway could be a viable therapeutic strategy for most cases of PTC.

We first tested the activity of these compounds in PCLC3 cells that conditionally express effector mutants signaling along the MAPK pathway. Both RAF inhibitors effectively blocked RET/PTC, RAS, and RAF-induced MEK and ERK activation. In
human thyroid cancer cell lines with RET or BRAF mutations, MEK and ERK were constitutively phosphorylated. The time course of inhibition of pMEK and pERK by AAL881 varied considerably between the cell lines. Whereas pMEK inhibition was sustained through 24 hours, in TPC1 and ARO cells inhibition of ERK phosphorylation was transient. A paradoxical feedback up-regulation of RAF-1 activity has been observed after treatment of cells with the RAF inhibitor ZM 336372, which was postulated to account for the inability of this compound to block epidermal growth factor–dependent ERK activation (28). However, the fact that AAL881 efficiently blocks MEK activation makes this an unlikely explanation for the rebound in ERK phosphorylation seen at later time points. Alternatively, the effects of the RAF inhibitors on expression of MAPK phosphatases may account for these findings. The activity of ERK1 and ERK2 is tightly regulated in a spatiotemporal manner that is critical to their biological function (29). Rapid inactivation of ERK1 and ERK2 after exposure to a mitogenic signal occurs through the action of constitutively expressed Ser/Thr (PP2A) or Tyr phosphatases (e.g., PTP-SL; ref. 30). The delayed phase of ERK inactivation is believed to require gene expression of dual-specificity MAPK phosphatases with activity on both Ser/Thr and Tyr (31–34). Of these, MKP-3 (35) and DUSP5 (36) have shown specificity for ERKs. Whereas MKP-3 is a cytoplasmic ERK phosphatase, DUSP5 is located in the nucleus (36). In mammalian cells, DUSP5 expression is inducible by heat shock and growth factors (37). By contrast, the regulation of MKP-3 expression is not clearly understood. Although MKP-3 expression seems to be constitutive in some cell types (38), in others MKP-3 expression is induced after exposure to growth factors (39, 40). In thyroid PCCL3 cells, both DUSP5 and MKP-3 gene expression are markedly induced by conditional activation of RET/PTC or BRAFV600E.2

Hence, the observation that RAF inhibitors down-regulate expression of MKP-3 and DUSP5 was to be expected and may explain the rebound in ERK phosphorylation. Although this needs to be formally proven, these findings raise the possibility that concomitant down-regulation of ERK phosphatases by RAF inhibitors could impair their efficacy in vivo.

Despite these theoretical concerns, both AAL881 and LBT613 were potent inhibitors of growth of all thyroid cancer cell lines in vitro. Of the four lines studied, one had a RET/PTC1 rearrangement and the other three harbored the BRAF mutation. AAL881, which inhibits RAF but not RET kinase activity, was ~10-fold more potent in decreasing growth of TPC1 cells than of the cell lines with BRAF mutation. We cannot exclude the possibility that AAL881 may have effects on other kinases that contribute to this differential action. LBT613 was also ~10-fold more potent in TPC1 cells than in all others. However, this compound is active on both RET and RAF kinase in vitro. Namba et al. (27) had previously reported that the MEK inhibitor U0126 was an effective inhibitor of cell growth and proliferation induced by CRAF kinase–induced lung adenoma, in which the MEK inhibitor CI-1040 decreased adenoma formation and cell proliferation induced by the CRAF transgene (41). Interestingly, the RAF inhibitor BAY 43-9006 was not effective in this model.

Both RAF inhibitors arrested cells in G1-G0 after short-term incubations. There was no sub-G1 peak after 48 hours of exposure to the inhibitors, indicating that RAF inhibition does not acutely activate an apoptotic pathway. Analysis of
single mutant ARAF or CRAF knockout mice did not support a role for these kinases in cell proliferation but instead suggested a MEK-independent role of CRAF in prevention of apoptosis (42–44). Knockdown of expression of oncogenic BRAF in human melanoma cell lines is associated with marked apoptosis, suggesting that this oncoprotein does confer a survival advantage under certain experimental conditions (45). However, combined knockout of CRAF and ARAF in mice is not associated with increased apoptosis in embryos. By contrast, double-knockout mouse tissues show decreased Ki67 staining and double-knockout mouse embryonic fibroblasts exhibit delayed entry into S phase after serum stimulation (46). Based on these findings, it would be predicted that pan-RAF inhibitors would have similar effects on growth and cell cycle progression. Indeed, at the concentrations of AAL881 and LBT613 that were sufficient to inhibit cell growth, there were only marginal effects on cell detachment and death in thyroid cancer cells, suggesting that the primary effect was through impairment of progression into S phase.

AAL881 and LBT613 inhibited growth of ARO and NPA cell xenografts. These compounds also seemed to hit their appropriate target in ARO cells in vivo as MEK and ERK phosphorylation were markedly reduced in the tumor tissues. Although the compounds also reduced NPA cell xenograft growth, there was no significant effect on MEK or ERK phosphorylation. Of note, AAL881 and LBT613 are dual inhibitors of KDR and the Raf family of kinases and efficacy in the NPA line in vivo could be the result of KDR inhibition. In xenograft efficacy models, KDR inhibition usually gives treated versus control values of 50%. This is consistent with the relatively weak effect of the RAF inhibitors on ERK activation in NPA cells in vitro. The cause of this relative resistance to RAF inhibition is unclear. The BRAF cDNA of NPA cells was sequenced and, in addition to the previously reported V600E mutation, was found to harbor a threonine-to-serine substitution at amino acid 119. Not much is known about this particular region of the NH2 terminus of BRAF but there is no reason to believe that it modulates BRAF kinase activity. Although a decrease in ERK phosphatase expression may have contributed to the resistance to RAF inhibitors, MKP-3 and DUSP5 expressions were similarly down-regulated in ARO cells.

In conclusion, small-molecule RAF-specific inhibitors inhibit growth of papillary thyroid cancer cells with mutations of RET or BRAF. Because of the strict lack of overlap of mutations of genes coding for MAPK pathway components in this tumor type, the RAF kinases seem particularly well suited as targets for therapy, even of advanced thyroid cancers that have progressed to a poorly differentiated state. We expect that the feasibility and efficacy of this approach will soon be tested in clinical trials.
Raf Inhibitors Block Thyroid Cancer Cell Growth

References


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