Targeting $BRAF^{V600E}$ in thyroid carcinoma: therapeutic implications

Constantine S. Mitsiades,1,2 Joseph Negri,1,2 Ciaran McMullan,1,2 Douglas W. McMillin,1,2 Elias Sozopoulos,4 Galinos Fanourakis,1,2,4 Gerassimos Voutsinas,5 Sophia Tseleni-Balafouta,4 Vassiliki Poulaki,3 David Batt,6 and Nicholas Mitsiades1,2

1Department of Medical Oncology, Dana-Farber Cancer Institute, 2Department of Medicine, Harvard Medical School, and 3Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts; 4Department of Pathology, University of Athens, 5Laboratory of Environmental Mutagenesis and Carcinogenesis, Institute of Biology, National Center of Scientific Research “Demokritos”, Athens, Greece; and 6Oncology Research, Novartis Institutes for BioMedical Research, Inc., Cambridge, Massachusetts

Abstract
B-Raf is an important mediator of cell proliferation and survival signals transduced via the Ras-Raf-MEK-ERK cascade. $BRAF$ mutations have been detected in several tumors, including papillary thyroid carcinoma, but the precise role of B-Raf as a therapeutic target for thyroid carcinoma is still under investigation. We analyzed a panel of 93 specimens and 14 thyroid carcinoma cell lines for the presence of $BRAF$ mutations and activation of the mitogen-activated protein/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway. We also compared the effect of a B-Raf small inhibitory RNA construct and the B-Raf kinase inhibitor AAL881 on both B-Raf wild-type and mutant thyroid carcinoma cell lines. We found a high prevalence of the T1799A (V600E) mutation in papillary and anaplastic carcinoma specimens and cell lines. There was no difference in patient age, B-Raf expression, Ki67 immunostaining, or clinical stage at presentation between wild-type and $BRAF^{V600E}$ specimens. Immunodetection of phosphorylated and total forms of MEK and ERK revealed no difference in their phosphorylation between wild-type and $BRAF^{V600E}$ patient specimens or cell lines. Furthermore, a small inhibitory RNA construct targeting the expression of both wild-type B-Raf and B-Raf$^{V600E}$ induced a comparable reduction of viability in both wild-type and $BRAF^{V600E}$ mutant cancer cells. Interestingly, AAL881 inhibited MEK and ERK phosphorylation and induced apoptosis preferentially in $BRAF^{V600E}$-harboring cells than wild-type ones, possibly because of better inhibitory activity against B-Raf$^{V600E}$. We conclude that B-Raf is important for the pathophysiology of thyroid carcinomas irrespective of mutational status. Small molecule inhibitors that selectively target B-Raf$^{V600E}$ may provide clinical benefit for patients with thyroid cancer. [Mol Cancer Ther 2007;6(3):1070–8]

Introduction
Thyroid cancer is the most prevalent endocrine malignancy and is diagnosed annually in ~25,700 new cases (~19,200 in women and 6,500 in men) in the U.S. alone (1). Surgical resection can be curative for those well-differentiated thyroid cancers detected prior to the establishment of local or distant metastases, however, >1,500 patients with thyroid cancer succumb to this disease each year. To address the critical need for novel and effective therapeutic strategies for aggressive and/or disseminated thyroid carcinomas, it is important to obtain a comprehensive insight into the signaling cascades which trigger the uncontrolled proliferation and/or resistance of thyroid carcinoma cells to proapoptotic stimuli.

Among the broad spectrum of signaling pathways with putative implications for the pathophysiology of thyroid carcinomas, significant emphasis has been placed on the RET/Ras/Raf/MEK/ERK cascade because mutational activation of Ras is present in a sizable proportion of thyroid carcinomas, specifically, and 1/3 of human neoplasias, in general (2). B-Raf, another key signaling effector of this pathway, is mutated in a significant subset of papillary thyroid carcinomas, as well as in several other malignancies, including melanomas, colon, and ovarian carcinomas (2–12). Mitsutake et al. recently showed that B-Raf is the key mediator of rearranged during transfection (RET)/papillary thyroid carcinoma (PTC)–induced extracellular signal-regulated kinase (ERK) phosphorylation in thyroid cells (13). Thyroid-specific expression of $BRAF^{V600E}$ induces goiter and invasive papillary carcinoma, which transitions to poorly differentiated carcinoma (14). The precise effect of $BRAF$ mutations in the clinical aggressiveness of thyroid carcinomas, as well as its potential role as a therapeutic target, is still under investigation.

In this study, we investigated the functional role of $BRAF$ mutations in thyroid carcinoma cells and the therapeutic implications of targeting B-Raf function with small inhibitory RNA (siRNA) constructs (protein depletion) and a small molecule inhibitor of the B-Raf kinase domain (enzymatic inhibition). We show that $BRAF$ mutational status in thyroid carcinomas does not correlate with...
increased mitogen-activated protein/ERK kinase (MEK) or ERK phosphorylation, proliferation rate or clinical stage at presentation. However, we found that irrespective of its wild-type or mutant status, B-Raf function is a legitimate therapeutic target for thyroid carcinoma cells (primarily papillary and anaplastic carcinoma) because siRNA-mediated depletion of B-Raf protein significantly suppresses the viability of thyroid carcinoma cells bearing either wild-type or mutant B-Raf alleles. Interestingly, a small molecule B-Raf kinase inhibitor suppressed MEK and ERK phosphorylation and cell viability preferentially in thyroid carcinoma cells with B-RafV600E compared with B-Rafwt, possibly due to a higher affinity for B-Raf V600E. These findings indicate that both mutated and wild-type B-Raf proteins function as cardinal mediators of proliferative/antiapoptotic signaling in thyroid cancer. At the same time, they also constitute proof-of-principle for the feasibility of small molecule–based preferential inhibition of a constitutively active oncogenic mutant kinase, but not its wild-type counterpart.

Materials and Methods

Human Tissues

Archival formalin-fixed and paraffin-embedded thyroid specimens from 93 patients with thyroid carcinomas, ages 20 to 74 years (mean ± SD, 46.7 ± 13.1), as well as seven benign follicular adenomas, were retrieved retrospectively from the files of the Pathology Department, University of Athens, Greece. They represented 58 papillary, 3 follicular, 5 oxyphilic (Huertthle), 7 anaplastic, and 20 medullary carcinomas. All thyroid specimens were removed from patients followed at the Endocrine Unit of the Evgenidion Hospital, Athens, Greece. All studies on patient material were conducted in accordance with the Declaration of Helsinki principles and institutional review board policies.

Cell Lines

Fourteen previously described thyroid carcinoma cell lines were used in this study. The papillary thyroid carcinoma cell lines BHP-2, BHP-5, BHP-7, BHP-10, BHP-14, BHP-17, BHP-18, and BHP-19 were generous gifts from Dr. Jerome M. Hershman (West Los Angeles Veterans Affairs Medical Center, Los Angeles, CA; refs. 15, 16). The SW579 cell line, derived from a poorly differentiated human thyroid adenocarcinoma (poorly differentiated carcinoma with nuclear features of papillary carcinoma and squamous differentiation), and the TT cell line, derived from a medullary thyroid carcinoma, were purchased from American Type Culture Collection (Manassas, VA). The papillary carcinoma cell line NPA, the anaplastic carcinoma cell lines FRO and ARO, and the follicular carcinoma cell line WRO were a generous gift from Dr. James A. Fagin (University of Cincinnati School of Medicine, Cincinnati, OH; ref. 17). All cells were grown in DMEM (BioWhittaker, Walkersville, MD) with 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 10% FCS (Life Technologies, Gaithersburg, MD), unless stated otherwise.

Reagents

The B-Raf inhibitor AAL881 (18, 19) was kindly provided by Dr. David Batt (Novartis Institutes for BioMedical Research, Inc., Cambridge, MA). The anti-Fas agonistic monoclonal antibody CH-11 was obtained from Panvera (Madison, WI). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and doxorubicin were from Sigma Chemical Co. (St. Louis, MO). The pan-caspase inhibitor ZVAD-FMK was purchased from Calbiochem (La Jolla, CA) and used at 20 μM/L.

Direct Sequencing

Genomic DNA from tissue samples were PCR-amplified using the following primers: B-Raf11F (5’-TCCCTCTCAGG-CATAAGGTA-3’) and B-Raf11R (5’-CGAACAGTGAATT-TTTCTTTGAT-3’; PCR product, 312 bp) for BRAF exon 11, and primers B-Raf15F (5’-TCATATGTCTTCTCTGA-TAGGA-3’) and B-Raf15R (5’-GGCCTAAAAATTTATCA-CGTGGA-3’; PCR product, 223 bp) for BRAF exon 15.

Cycle sequencing of the purified PCR products was carried out with one of the PCR primers using the Big dye terminator sequencing kit (Applied Biosystems, Foster City, CA). The Sephadex G-50–purified cycle sequencing products were analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

MTT Colorimetric Survival Assay

Cell viability was quantified using the MTT colorimetric assay, as previously described (20), and expressed as a percentage of the value of untreated controls. All experiments were repeated at least thrice, and each experimental condition was repeated in at least quadruplicate wells in each experiment. Data reported were average values ± SD of representative experiments.

Immunoblotting Analysis

Immunoblotting analysis was done as previously described (21). The antibodies used to detect phospho-MEK, total MEK, phospho-ERK p44/42, and total ERK p44/42 were from Cell Signaling (Beverly, MA). Antibodies for the detection of B-Raf and caspase-3 were from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody for PARP was from Biomol (Plymouth Meeting, PA).

Immunohistochemistry

Immunohistochemistry was done and evaluated as previously described (21). The antibodies used to detect phospho-MEK, total MEK, phospho-ERK p44/42, and total ERK p44/42 were from Cell Signaling; anti-B-Raf was from Santa Cruz Biotechnology; and anti-Ki67 was from Dako (Carpinteria, CA). The peroxidase reaction was developed with 3,3’-diaminobenzidine and the slides were counterstained with hematoxylin. The intensity of positive staining was evaluated on a scale of 0 to 3 (0 and 3 corresponded to the absence and highest degree of staining, respectively) with a 25× lens by an expert thyroid pathologist (S. Tseleni-Balafouta) who was blinded to the B-Raf mutational status of the specimens.

Suppression of B-Raf Expression by Transfection with B-Raf siRNA

To generate siRNA-B-Raf, two oligonucleotides consisting of ribonucleosides with the additional presence of
Table 1. Summary of sequencing results [wild-type (WT) or mutant state] of BRAF exons 11 and 15 in 14 thyroid carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Histologic subtype</th>
<th>BRAF status</th>
<th>Exon 11</th>
<th>Exon 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARO</td>
<td>Anaplastic</td>
<td>WT</td>
<td>WT</td>
<td>Mutant</td>
</tr>
<tr>
<td>FRO</td>
<td>Anaplastic</td>
<td>WT</td>
<td>WT</td>
<td>Mutant</td>
</tr>
<tr>
<td>WRO</td>
<td>Follicular</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>TT</td>
<td>Medullary</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>BHP 2</td>
<td>Papillary</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>BHP 5</td>
<td>Papillary</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>BHP 7</td>
<td>Papillary</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>BHP 10</td>
<td>Papillary</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>BHP 14</td>
<td>Papillary</td>
<td>WT</td>
<td>WT</td>
<td>Mutant</td>
</tr>
<tr>
<td>BHP 17</td>
<td>Papillary</td>
<td>WT</td>
<td>WT</td>
<td>Mutant</td>
</tr>
<tr>
<td>BHP 18</td>
<td>Papillary</td>
<td>WT</td>
<td>WT</td>
<td>Mutant</td>
</tr>
<tr>
<td>BHP 19</td>
<td>Papillary</td>
<td>WT</td>
<td>WT</td>
<td>Mutant</td>
</tr>
<tr>
<td>NPA</td>
<td>Papillary</td>
<td>WT</td>
<td>WT</td>
<td>Mutant</td>
</tr>
<tr>
<td>SW-579</td>
<td>Papillary</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

NOTE: All BRAF mutations detected were T1799A (V600E) mutations.

2'-deoxyribonucleosides at the 3' end, 5'-r(CGAGACC-GAUCUCUCAUCAG)d(TT)-3' and 5'-r(CGAGACC-GAUCGGUCUCG)d(TT)-3' were synthesized and annealed (Qiagen-Xeragon, Inc., Valencia, CA). This siRNA targets outside the V600E mutation site and thus abrogates the expression of both B-Raf<sup>V600E</sup> and B-Raf<sup>wt</sup>. Cells were exposed to 100 nmol/L of siRNA<sub>B-Raf</sub> in the presence of Transmessenger (Qiagen) for 4 h, according to the instructions of the manufacturer. Control experiments were done using nonsilencing siRNA (directed against Thermotoga maritimia mRNA; Qiagen).

Annexin V-Propidium Iodide Staining for Detection of Apoptosis

To confirm the induction of apoptosis in thyroid carcinoma cells treated with AAL881, we used the Annexin V-FLUOS kit (Roche Applied Science, Indianapolis, IN), according to the instructions of the manufacturer. BHP-14 cells were treated for 72 h with AAL881 (5 μmol/L) or vehicle, and then stained with propidium iodide (PI) and FITC-labeled Annexin V, and analyzed by dual-color flow cytometry. Cells that were Annexin V-FITC(+) [with translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane] and PI(−) [with intact cellular membrane] were considered as early apoptotic cells.

Statistical Analysis

To evaluate potential differences in age between patients with versus without BRAF mutation, we did independent samples’ t test. Fisher’s exact test was used to compare the frequency of BRAF mutations in male versus female patients. Pearson’s χ² test was done to evaluate whether the presence of BRAF mutation in papillary thyroid carcinoma tumor specimens correlates with intensity of immunostaining for B-Raf, Ki67, and phosphorylated and total forms of MEK and p42/44 ERK, or with disease stage (as defined by the American Joint Committee on Cancer tumor-node-metastasis classification, 6th edition). IC<sub>50</sub>s were calculated using STATISTICA software (StatSoft, Tulsa, OK). In all analyses, P < 0.05 was required for statistical significance.

Results

BRAF Mutations are Common among Both Primary Papillary Thyroid Tumors and Thyroid Cell Lines of Papillary Origin

We first investigated the prevalence of BRAF mutations in exons 11 and 15 (which correspond to the mutation hotspots in the B-Raf kinase domain) in a panel of paraffin-embedded thyroid tumor specimens and benign follicular adenomas, as well as in a series of thyroid carcinoma cell lines. We found a high prevalence of BRAF mutations in both primary and metastatic tumors, as well as in thyroid cell lines. We detected BRAF mutations in tumor cells from 22 of 93 patients (23.7%) and in 8 of 14 thyroid carcinoma cell lines. All BRAF mutations found were T→A transversions at position 1799 (T1799A), which corresponds to amino acid change of valine to glutamate in codon 600 (V600E). BRAF<sup>V600E</sup> was detected in the papillary cell lines BHP-5, BHP-14, BHP-17, BHP-18, BHP-19, and NPA, and the anaplastic cell lines ARO and FRO (Table 1). Several of these cell lines have been previously examined for BRAF mutations by other groups, with the same results (2, 9).

Among patients with thyroid cancer, BRAF<sup>V600E</sup> was found in 21 of 58 (36.2%) papillary carcinoma cases, 1 of 7 cases of anaplastic carcinoma, and in none of the 20 medullary, 3 follicular, 5 Hurthle cell carcinomas, or 7 benign follicular adenomas (Table 2). All mutations found were somatic, i.e., they were absent from corresponding nonmalignant tissue from the same patient, including in the case of two pairs of siblings (four patients) with papillary carcinoma in which we detected BRAF<sup>V600E</sup> mutations in all four carcinomas but not in normal thyroid tissue samples from these patients. This indicates that BRAF<sup>V600E</sup> alleles in thyroid carcinomas are derived from somatic mutational events and not inheritance of a germ line mutation. This

Table 2. Sequencing results from paraffin-embedded primary thyroid carcinoma specimens with B-Raf<sup>wt</sup> and B-Raf<sup>V600E</sup>, stratified according to histologic classification

<table>
<thead>
<tr>
<th>BRAF status</th>
<th>Wild-type (%)</th>
<th>Mutated (%)</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillary</td>
<td>37 (63.8%)</td>
<td>21 (36.2%)</td>
<td>58</td>
</tr>
<tr>
<td>Follicular</td>
<td>3 (100%)</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>Medullary</td>
<td>20 (100%)</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>Anaplastic</td>
<td>6 (85.7%)</td>
<td>1 (14.3%)</td>
<td>7</td>
</tr>
<tr>
<td>Hurthle</td>
<td>5 (100%)</td>
<td>—</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>71 (76.3%)</td>
<td>22 (23.7%)</td>
<td>93</td>
</tr>
</tbody>
</table>

NOTE: All BRAF mutations detected were T1799A (V600E) mutations.
suggests that BRAF<sup>V600E</sup> does not seem to be the oncogene responsible for familial papillary thyroid carcinoma.

Within the population of papillary thyroid carcinomas, there was a trend for higher frequency of BRAF<sup>V600E</sup> in male versus female patients, but it did not reach statistical significance: 10 of 20 (50.0%) male patients harbored BRAF<sup>V600E</sup> versus 11 of 38 (28.9%) female patients (P = 0.153; Table 3). There was also no correlation between BRAF<sup>V600E</sup> presence and a higher disease stage according to the tumor-node-metastasis classification (P = 0.713; Table 3). We found no significant association of the BRAF mutational status with the age of patients with papillary thyroid cancer (mean age ± SD, 45.9 ± 8.7 versus 42.8 ± 13.3 for patients with BRAF<sup>V600E</sup> versus BRAF<sup>wt</sup>; P = 0.297).

**Table 3. Stratification according to tumor-node-metastasis stage among papillary thyroid carcinoma patients with B-Raf<sup>wt</sup> versus B-Raf<sup>V600E</sup>**

<table>
<thead>
<tr>
<th>BRAF status</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (%)</td>
<td>Mutated (%)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>19 (65.5%)</td>
</tr>
<tr>
<td>II</td>
<td>7 (70.0%)</td>
</tr>
<tr>
<td>III</td>
<td>6 (50.0%)</td>
</tr>
<tr>
<td>IV</td>
<td>5 (71.4%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>27 (71.1%)</td>
</tr>
<tr>
<td>Male</td>
<td>10 (50.0%)</td>
</tr>
</tbody>
</table>

NOTE: All BRAF mutations detected were T1799A (V600E) mutations.

The Presence of BRAF<sup>V600E</sup> Does Not Correlate with Stronger Activation of the MEK/ERK Pathway or Higher Proliferation Rate in Thyroid Carcinoma Cells

We next evaluated the effect of B-Raf<sup>V600E</sup> on downstream signaling effectors of B-Raf, such as MEK and p42/44 ERK, which are activated via phosphorylation by B-Raf and MEK itself, respectively. There was no difference between BRAF<sup>wt</sup> and BRAF<sup>V600E</sup> tumor specimens in the immunostaining for total MEK (P = 0.503), phospho-MEK (P = 0.851), total p44/42 ERK (P = 0.841), phospho-p44/42 ERK (P = 0.179; Fig. 1A–D), or Ki67 (P = 0.660). Moreover, there was no difference in B-Raf immunostaining between BRAF<sup>wt</sup> and BRAF<sup>V600E</sup> specimens (data not shown). Both ERK and MEK immunostaining were very rare (<5% of cells positive) in nonmalignant thyroid tissue, with the exception of areas affected by intense lymphocytic infiltration.

Furthermore, the degree of phosphorylation of MEK and ERK in thyroid carcinoma cell lines were calculated by densitometric analysis of the immunoblotting films. Both the ratios pMEK/total MEK and pERK/total ERK did not show any significant difference between BRAF<sup>wt</sup> and BRAF<sup>V600E</sup> cell lines incubated in serum-free medium for 24 h (Fig. 1E). Taken together, these data support that the presence of B-Raf<sup>V600E</sup> was not correlated with increased activation of the MEK/ERK signaling pathway in thyroid carcinoma cells.

**Inhibition of B-Raf Expression Lowers Viability in both B-Raf<sup>wt</sup> and B-Raf<sup>V600E</sup> Cell Lines**

Based on the high prevalence of BRAF<sup>V600E</sup> in thyroid carcinoma specimens and cell lines, we hypothesized that...
B-Raf expression would be crucial in at least a subset of thyroid carcinomas. To deplete cells of B-Raf protein (both wild-type and mutant), we transfected a panel of thyroid carcinoma cell lines with a BRAF siRNA (targeting outside the V600E mutation site). Seventy-two hours later, cell viability was lower in cells transfected with BRAF siRNA than in those transfected with the nonsilencing control siRNA. This effect was comparable in the BRAF wt (WRO and BHP-7) and the BRAF V600E (FRO and BHP-14) cell lines. A, immunoblotting analysis of BHP-14 cells confirms the suppression of B-Raf expression by the pan-BRAF–specific siRNA, but not the nonsilencing RNA (immunoblotting for tubulin confirms equal protein loading).

A Small Molecule B-Raf Inhibitor (AAL881) Exhibits Partial Selectivity for BRAF V600E Cells

We next investigated the effect of the small molecule B-Raf kinase inhibitor AAL881 on a panel of wild-type and mutant (V600E) thyroid carcinoma cell lines (72 h of incubation in serum-free conditions). All BRAF V600E thyroid carcinoma cell lines exhibited an IC_{50} to AAL881 of ≤5 μmol/L. The IC_{50} of AAL881 for BRAF wt cell lines was >15 μmol/L, which suggests that B-Raf kinase inhibitors such as AAL881 can selectively target tumor cells expressing B-Raf V600E and thus potentially spare normal cells which harbor only B-Raf wt (Fig. 3A).

AAL881 Inhibits Phosphorylation of MEK and ERK Specifically in BRAF V600E Thyroid Carcinoma Cells

Having shown a relative selectivity of the antitumor effect of AAL881 for BRAF V600E versus BRAF wt thyroid cell lines, we evaluated the effect of AAL881 on downstream signaling effectors of B-Raf, such as MEK and p42/44 ERK. Following overnight serum starvation, cells were either treated with vehicle or AAL881 in serum-free conditions. Although AAL881 (5 μmol/L) rapidly and potently suppressed MEK and ERK phosphorylation in BRAF V600E BHP14 cells, it had only a minor and transient effect in BRAF wt WRO cells (Fig. 3B). This suggests that MEK and ERK activation was significantly more sensitive to inhibition by AAL881 in BRAF V600E than in BRAF wt cells.

AAL881 Induces Apoptosis in BRAF V600E Thyroid Carcinoma Cells

We characterized the nature of the effect of AAL881 on thyroid carcinoma cells. We found that AAL881 (5 μmol/L for 72 h) induced the cleavage of caspase-3 and PARP in BRAF V600E BHP-14 cells. This finding is in agreement with the recently shown ability of AAL881 to induce apoptosis in human glioma cell lines in vitro (19).

**Figure 3.** The B-Raf inhibitor AAL881 preferentially targets BRAF V600E cell lines. A, a panel of four BRAF wt (SW579, ①; WRO, ②; BHP10, ③; BHP2, ④) and seven BRAF V600E (FRO, ⑤; BHP14, ⑥; NPA, ⑦; BHP19, ⑧; BHP18, ⑨; BHP5, ⑩; and BHP17, ⑪) thyroid carcinoma cell lines were treated with the small molecule B-Raf kinase inhibitor AAL881 (0–10 μmol/L) in serum-free DMEM for 72 h. AAL881 had a profound negative effect on the viability of BRAF V600E cell lines. All BRAF V600E thyroid cell lines exhibited an IC_{50} to AAL881 of ≤5 μmol/L (in contrast to IC_{50} values of >15 μmol/L in the BRAF wt cell lines examined). Cell viability was quantified with MTT colorimetric survival assays and expressed as % surviving drug-treated cells over control cells (mean ± SD). Cell viability was quantified with MTT colorimetric survival assays and expressed as % surviving drug-treated cells over control cells (mean ± SD). B, AAL881 (5 μmol/L) rapidly and potently suppresses MEK and ERK phosphorylation in BRAF V600E BHP-14 cells, but has only a minor and transient inhibitory effect in BRAF wt WRO cells. All cells were treated with vehicle or AAL881 in serum-free conditions after overnight serum starvation.
V(+)/PI(+) cells, which represent late apoptotic and necrotic cells.

compared with controls (2.5%). There was also an increase in the Annexin phenomenon.

functional role of caspases and the apoptotic nature of this phenomenon.

AAL881-induced cell death (Fig. 4D), confirming the functional role of caspases and the apoptotic nature of this phenomenon.

Figure 4. The B-Raf inhibitor AAL881 induces caspase-dependent apoptosis in BRAFV600E cell lines A, BHP-14 cells treated with AAL881 (5 μmol/L for 72 h) exhibit typical cleavage of PARP and caspase-3, suggesting the presence of apoptosis. B and C, BHP-14 cells treated with AAL881 (5 μmol/L for 72 h), C or vehicle (B). Representative double staining for Annexin-FITC/PI(+) indicates a significant induction of apoptotic cell death [Annexin V(+)/PI(−) cells] in the AAL881-treated cells (26.6%) compared with controls (2.5%). There was also an increase in the Annexin V(+)/PI(+) cells, which represent late apoptotic and necrotic cells. D, treatment of BHP-14 cells with the pan-caspase inhibitor ZVAD-FMK (20 μmol/L) had a strong attenuating effect on cell death induced by AAL881 (10 μmol/L for 72 h; P < 0.0001), confirming the functional role of caspases and the apoptotic nature of this phenomenon.

To further confirm that AAL881 induces apoptosis in thyroid carcinoma cells, we did double Annexin V/PI labeling. Annexin V binds with high affinity to the phospholipid phosphatidylserine, which translocates from the inner to the outer leaflet of the cell membrane during apoptosis. Because PI cannot cross the intact cell membrane, cells that are PI(−) yet Annexin V(+) represent early apoptotic cells that still maintain membrane integrity. In BHP-14 cells treated with AAL881 (5 μmol/L) or vehicle for 72 h, and then stained with PI and FITC-labeled Annexin V, there was a significant increase in the Annexin V(+)/PI(−) cell population (26.6%, compared with 2.5% in control cells; Fig. 4B and C).

Treatment of BHP-14 cells with the pan-caspase inhibitor ZVAD-FMK (20 μmol/L) had a strong attenuating effect on AAL881-induced cell death (Fig. 4D), confirming the functional role of caspases and the apoptotic nature of this phenomenon.

AAL881 Sensitizes Thyroid Carcinoma Cells to Fas-Mediated Apoptosis

We then studied the effect of AAL881 on thyroid cancer cell responsiveness to other proapoptotic stimuli. We specifically focused on the effect of AAL881 on Fas-mediated apoptosis because thyroid carcinoma cells are resistant to Fas receptor cross-linking (21). A short incubation with AAL881 (5 μmol/L for 6 h pretreatment) sensitized BHP-14 cells to Fas stimulation (treatment with 500 ng/mL of the Fas cross-linking antibody CH11 for an additional 16 h; Fig. 5B). Consistent with its sensitizing effect on Fas-mediated apoptosis, AAL881 treatment of BHP-14 cells induced up-regulation of Fas expression and down-regulated the intracellular levels of the caspase-8 inhibitor FLIP (Fig. 5B), a known suppressor of Fas-mediated apoptosis (22–25). These results not only reinforce the notion that B-Raf kinase activity regulates thyroid tumor cell resistance to apoptosis, but also that small molecule inhibitors of the B-Raf kinase sensitize thyroid cancer cells to other proapoptotic antitumor agents.

Discussion

In this study, we confirm that BRAF mutations are highly prevalent in thyroid carcinomas, specifically of the papillary and anaplastic histologic subtype (both primary tumor specimens and cell lines), and investigate their pathophysiologic significance for the neoplastic cell. All detected mutations were BRAFV600E. Their presence did not correlate with higher activation of the MEK/ERK pathway, higher proliferative rate, or advanced disease stage among patients with papillary thyroid carcinoma. siRNA-mediated depletion of B-Raf protein significantly suppressed the viability of thyroid carcinoma cells irrespective of BRAFV600E status. Interestingly, the small molecule B-Raf kinase inhibitor AAL881 suppressed MEK and ERK phosphorylation and induced apoptosis preferentially in thyroid carcinoma cells with BRAFV600E, possibly due to higher affinity for B-RafV600E than B-Rafwt. AAL881 also upregulated the expression of the death receptor Fas, downregulated the expression of the caspase-8 inhibitor FLIP, and sensitized the thyroid carcinoma cells to apoptosis induced by Fas cross-linking. BRAF-activating somatic mutations are present in the majority of human malignant melanomas and in subsets of thyroid, colon, and ovarian carcinomas (2–11, 26). The overwhelming majority of these mutations are single nucleotide thymine-to-adenine (T→A) transversions at nucleotide 1799, resulting in a valine to glutamic acid change at amino acid 600 (V600E) within the activation segment of B-Raf. B-RafV600E assumes a three-dimensional conformation mimicking the B-Raf phosphorylation at Thr599 and Ser602 residues (which corresponds to the activated state of B-Raf), resulting in constitutive activation of B-Raf and its downstream targets. In agreement with previous findings from several groups (2, 7, 9), BRAF mutations were present only in papillary and anaplastic tumors. No mutations were detected in medullary
expression and down-regulated the intracellular levels of FLIP. AAL881 treatment of BHP-14 cells induced up-regulation of Fas (72 h). Consistent with its sensitizing effect on Fas-mediated apoptosis, it did sensitize them to Fas-mediated apoptosis. B, Immunoblotting for the apoptosis-inducing receptor Fas and the antiapoptotic protein FLIP, a caspase-8 inhibitor FLIP and known suppressor of Fas cross-linking–induced apoptosis, in BHP-14 cells treated with AAL881 (5 μmol/L for 72 h). Consistent with its sensitizing effect on Fas-mediated apoptosis, AAL881 treatment of BHP-14 cells induced up-regulation of Fas expression and down-regulated the intracellular levels of FLIP.

Figure 5. The B-Raf inhibitor AAL881 sensitizes thyroid carcinoma cells to Fas-mediated apoptosis. A, BHP-14 cells were briefly pretreated with AAL881 (5 μmol/L for 6 h pretreatment), followed by treatment with 500 ng/mL of the Fas cross-linking antibody CH11 for 16 h. At the end of these incubations, cell survival was quantified by MTT. This exposure to AAL881 was too short to have any negative effect on BHP-14 cells, but it did sensitize them to Fas-mediated apoptosis. B, Immunoblotting for the apoptosis-inducing receptor Fas and the antiapoptotic protein FLIP, a caspase-8 inhibitor FLIP and known suppressor of Fas cross-linking–induced apoptosis, in BHP-14 cells treated with AAL881 (5 μmol/L for 72 h). Consistent with its sensitizing effect on Fas-mediated apoptosis, AAL881 treatment of BHP-14 cells induced up-regulation of Fas expression and down-regulated the intracellular levels of FLIP.

carcinoma cells, a finding not surprising, given their different histologic origin. We, therefore, excluded medullary thyroid carcinoma cells from subsequent in vitro experiments in our study. However, the lack of BRAF mutations in follicular thyroid carcinomas is indeed surprising, as these cells originate from the follicular thyroid epithelium, just like papillary and anaplastic carcinomas. Because B-Raf is a downstream mediator of Ras signaling and because mutationally activated forms of Ras proteins are present in approximately one-third of all human cancers (27), the detection of mutant BRAF alleles has been viewed as a key evidence in support of the role of constitutive Ras/Raf/ERK signaling in the establishment and/or biological behavior of these tumors. However, the role of the BRAF V600E mutation in thyroid carcinoma progression remains unclear.

Although some studies have suggested that the BRAF V600E mutation is associated with extrathyroidal invasion, lymph node metastasis, advanced tumor stage, and tumor recurrence (7, 28, 29), several other studies have failed to detect such an association (30–35). The reason for this discrepancy is unknown. We would like to point out that, in papillary thyroid carcinomas, lymph node metastasis frequently occurs even in well-differentiated tumors that behave clinically in a relatively indolent manner, and does not carry the grave prognostic significance that it does in other solid malignancies. Therefore, it may not be the best surrogate marker of clinical aggressiveness. In other neoplasias, such as melanoma, the prognostic significance of BRAF mutations has also been questioned, especially because they are frequently detected in benign/premalignant cutaneous lesions (e.g., nevi; refs. 36–38). Similarly, the BRAF V600E mutation has been frequently found in microcarcinomas of the thyroid (30), which suggests a role in tumor initiation rather than tumor progression. On the other hand, BRAF mutations are also found in poorly differentiated and anaplastic thyroid carcinomas, suggesting that they may play a role in their highly aggressive behavior (7, 11, 39, 40). In the study of Nikiforova et al. (7), all BRAF-mutant poorly differentiated and anaplastic carcinomas contained areas of preexisting papillary carcinoma, and the BRAF V600E mutation was present in both the well-differentiated and dedifferentiated components, suggesting that it may have contributed to the transition from PTC to anaplastic carcinoma. In support of this hypothesis, thyroid-specific expression of BRAF V600E induces goiter and invasive PTC, which transitions to poorly differentiated carcinomas (14).

Interestingly, the studies thus far have not shown an association of the BRAF V600E mutation with larger size of the primary tumor (41). This suggests that the effect of BRAF V600E on tumor aggressiveness, if any, is mediated via increased metastatic potential and not via increased proliferation of the neoplastic cells. This is supported by the recent finding that B-Raf V600E preferentially induces metalloproteinase expression (42). In our present study, we did not detect any association between the BRAF V600E mutation and disease stage at presentation or tendency to give lymph node metastasis. In agreement, we did not observe increased immunostaining for the proliferation marker Ki67 or the phosphorylated forms of MEK and p42/44 ERK (downstream signaling mediators of the Ras/Raf pathway) in BRAF V600E compared with BRAF wt papillary thyroid carcinoma specimens and cell lines.

Recently, Salvatore et al. reported that depletion of B-Raf protein by RNA interference exerts an antiproliferative effect on BRAF V600E thyroid carcinoma cells (43). Also, Ouyang et al. showed that the Raf kinase inhibitors AAL881 and LBT-613 suppress the growth of BRAF V600E thyroid carcinoma cells (18). We extended these studies to compare the effect of protein depletion and enzymatic inhibition between BRAF wt and BRAF V600E thyroid carcinoma cell lines. We found that a siRNA construct which specifically targets an area of the BRAF gene outside the V600E mutation site (thus equally suppressing both B-Raf wt and B-Raf V600E protein expression) led to a comparable decrease in viability of both BRAF wt- and BRAF V600E-bearing thyroid carcinoma cell lines in vitro, supporting an important role for B-Raf in thyroid carcinoma cell viability irrespective of wild-type or mutant status. This can potentially be attributed to the fact that the activity of
B-Raf is determined not only by its intrinsic enzymatic activity (which is higher in B-RafV600E than in its wild-type counterpart; refs. 3–6), but also by the degree of its stimulation by upstream signaling regulators. This suggests that alternative molecular lesions upstream of B-Raf, e.g., mutant RAS or RET/PTC translocations (44), or autocrine growth factor signaling (45), can sustain a high level of stimulation of B-Raf activity, even in the absence of constitutively activating V600E mutational events, leading to comparable stimulation of downstream kinases (MEK/ERK) and proliferation rate. Specifically, Mitsutake et al. have shown that B-Raf mediates RET/PTC-induced mitogen-activated protein kinase activation in thyroid cells (13), whereas Mesa et al. showed that expression of a substantial percentage of RET/PTC3-regulated genes was B-Raf-dependent (42). As RET/PTC, RAS, and B-Raf mutational activation have been shown to be frequently present yet mutually exclusive in thyroid carcinomas (2), the constitutive proliferative/antiapoptotic activation by all these events is eventually mediated via B-Raf (13), which suggests that, irrespective of its mutational status, B-Raf is a valid therapeutic target for this broader spectrum of molecularly defined thyroid carcinomas.

To obtain further insight into how B-Raf function can be therapeutically targeted, we expanded on our B-Raf inhibition studies using the small molecule B-Raf kinase inhibitor AAL881 (18, 19). AAL881 had more potent antitumor activity against B-RafV600E than B-Rafwt cell lines, and in further confirmation of its selective effect on mutant B-Raf, AAL881 suppressed MEK and ERK phosphorylation more efficiently in B-RafV600E cell lines, suggesting higher affinity of this inhibitor for the three-dimensional structure of the mutant kinase domain. This selectivity is supported by early data on the inhibition of enzymatic activity of various Raf forms by AAL881 (IC50 for enzymatic activity of B-RafV600E and B-Rafwt was 0.22 and 0.94 μM/L, respectively; ref. 18). This represents a paradigm for an alternative therapeutic approach, in which selectively targeting the mutant kinase form lowers the risk of side effects related to inhibition of the wild-type kinase in normal cells, at the expense of narrowing the therapeutic spectrum of such an inhibitor only to cancers harboring the respective mutation.

Our findings suggest two distinct approaches for the design and potential clinical development of small molecule kinase inhibitors against B-Raf. On one hand, B-Raf kinase activity is important for the pathophysiology of all types of papillary and anaplastic thyroid carcinomas, even those with B-Rafwt, suggesting that a pan-B-Raf inhibitor could be effective against all such carcinomas. On the other hand, small molecule inhibitors with relative specificity for mutated kinases can be a useful strategy to selectively target tumor cells harboring the mutation whereas sparing normal cells expressing the wild-type kinase. Furthermore, this setting could constitute a prototype for the implementation of individualized, “patient-specific,” molecular targeted therapy, in which the molecular analysis for V600E BRAF mutation could potentially guide the management of patients with thyroid carcinoma towards receiving a kinase inhibitor with higher specificity against the mutant BRAF form versus other therapies directed against the wild-type form and/or other upstream levels of regulation of Raf activity.

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References


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Constantine S. Mitsiades, Joseph Negri, Ciaran McMullan, et al.


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