BRAF Is a Therapeutic Target in Aggressive Thyroid Carcinoma

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Abstract

Purpose: Oncogenic conversion of BRAF occurs in ~44% of papillary thyroid carcinomas and 24% of anaplastic thyroid carcinomas. In papillary thyroid carcinomas, this mutation is associated with an unfavorable clinicopathologic outcome. Our aim was to exploit BRAF as a potential therapeutic target for thyroid carcinoma.

Experimental Design: We used RNA interference to evaluate the effect of BRAF knockdown in the human anaplastic thyroid carcinoma cell lines FRO and ARO carrying the BRAF V600E (V600E-BRAF) mutation. We also exploited the effect of BAY 43-9006 [N-(3-trifluoromethyl-4-chlorophenyl)-N’-(4-[(2-methylcarbamoyl pyridin-4-yl)oxyphenyl])urea], a multitarget inhibitor able to inhibit RAF family kinases in a panel of six V600E-BRAF-positive thyroid carcinoma cell lines and in nude mice bearing ARO cell xenografts. Statistical tests were two sided.

Results: Knockdown of BRAF by small inhibitory duplex RNA, but not control small inhibitory duplex RNA, inhibited the mitogen-activated protein kinase signaling cascade and the growth of ARO and FRO cells (P < 0.0001). These effects were mimicked by thyroid carcinoma cell treatment with BAY 43-9006 (IC50 = 0.5-1 μmol/L; P < 0.0001), whereas the compound had negligible effects in normal thyrocytes. ARO cell tumor xenografts were significantly (P < 0.0001) smaller in nude mice treated with BAY 43-9006 than in control mice. This inhibition was associated with suppression of phospho–mitogen-activated protein kinase levels.

Conclusions: BRAF provides signals crucial for proliferation of thyroid carcinoma cells spontaneously harboring the V600E-BRAF mutation and, therefore, BRAF suppression might have therapeutic potential in V600E-BRAF-positive thyroid cancer.

Thyroid tumors are the most frequent neoplasms of the endocrine system (1). Well-differentiated thyroid carcinomas account for >90% of all thyroid cancers and include papillary and follicular carcinomas. Papillary thyroid carcinoma is the most prevalent subtype. Although papillary thyroid carcinoma is usually curable with surgery and adjuvant radioiodine treatment, some patients may show an aggressive disease and lose radioiodine concentration ability. Papillary thyroid carcinoma subtypes, like the tall-cell variant, more frequently have an aggressive behavior (2). Undifferentiated (anaplastic) thyroid carcinoma accounts for ~2% to 5% of all thyroid cancers (3). Despite its rarity, more than half of the deaths attributed to thyroid cancer result from anaplastic thyroid carcinoma (3–5). More than 25% of anaplastic thyroid carcinoma patients have coincidentally detected well-differentiated carcinoma, suggesting that, at least in some cases, anaplastic thyroid carcinoma derives from a preexisting well-differentiated carcinoma (3–5). Rapid growth and dissemination characterize the clinical course of anaplastic thyroid carcinoma. Virtually, all anaplastic thyroid carcinoma patients die from their disease in 2 to 7 months; death is attributable to upper airway obstruction and suffocation in half of the patients (3–5). Anaplastic thyroid carcinomas do not concentrate radioiodine and do not respond to conventional chemotherapy (3–5).

BRAF belongs to the RAF family of serine/threonine kinases. RAF proteins are components of the RAF-MEK [mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase]-ERK pathway, a highly conserved signaling module in eukaryotes. They are activated through binding to RAS in its GTP-bound state. Once activated, RAF kinases phosphorylate MEK, which in turn phosphorylates and activates ERK (6). Activation of BRAF has emerged as the most prevalent oncogenic mutation in thyroid carcinoma (7–14). Overall, this genetic alteration is found in ~44% of papillary thyroid carcinoma and 24% of anaplastic thyroid carcinoma (reviewed in ref. 15). In the case of anaplastic thyroid carcinoma, BRAF mutations are restricted to those cases that...
arose in association with papillary thyroid carcinoma (12, 14). A transversion from thymine to adenine (T1799A), leading to a Glu for Val substitution at residue 600 (V600E), accounts for >90% of BRAF mutations in thyroid carcinomas. Other more rare mutations have been described (reviewed in ref. 15). The V600E mutation enhances BRAF activity by disrupting the autoinhibited state of the kinase (16). Another interesting mechanism for BRAF activation has been described in radiation-induced papillary thyroid carcinoma, where a paracentric inversion of chromosome 7q resulted in the in-frame fusion between the AKA9 gene and BRAF (17).

Consistent with a pivotal role in thyroid cancer initiation, \textsuperscript{V600E}BRAF has been found in microcarcinomas (15), and it was shown to induce transformed features in thyroid follicular cells in culture (18, 19) and thyroid carcinoma formation in transgenic mice (20). Many evidences suggest that \textsuperscript{V600E}BRAF plays a role in thyroid cancer progression as well: (a) Adoptive expression of \textsuperscript{V600E}BRAF induces genomic instability in cultured thyrocytes (19); (b) thyroid tumors in \textsuperscript{V600E}BRAF-transgenic undergo dedifferentiation and metastasis formation (20); and (c) papillary thyroid carcinoma with the \textsuperscript{V600E}BRAF mutation often presents with extrathyroidal invasion, lymph node metastasis, and advanced tumor stage (14). Importantly, the \textsuperscript{V600E}BRAF mutation was frequently associated to loss of p13-avdity and papillary thyroid carcinoma recurrence (14).

In this framework, BRAF could be an appealing therapeutic target for thyroid carcinomas, especially for aggressive papillary thyroid carcinoma subtypes and anaplastic thyroid carcinoma. Here, we show that suppression of BRAF expression exerts cytostatic activity in \textsuperscript{V600E}BRAF-positive thyroid carcinoma cell lines. Moreover, we show that BAY 43-9006 [N-(3-trifluoromethyl-4-chlorophenyl)-N’-(4-(2-methylcarbamoyl pyridin-4-yl)oxyphenyl)urea], a multikinase ATP-competitive inhibitor able to obstruct RAF kinases (21–24), reduces tumor growth in an anaplastic thyroid carcinoma xenograft model.

Materials and Methods

Compounds. BAY 43-9006 was provided by Bayer HealthCare Pharmaceuticals (West Haven, CT). For \textit{in vitro} experiments, BAY 43-9006 was dissolved in DMSO. For \textit{in vivo} experiments, BAY 43-9006 was dissolved in Cremophor EL/ethanol alcohol (50:50; Sigma Cremophor EL, 95% ethyl alcohol; Sigma Chemical Co., St. Louis, MO) at 4-fold (4\times) the highest dose, foil-wrapped, and stored at room temperature. A fresh supply of the 4\times stock solution was prepared every 3 days. Final dosing solutions were prepared on the day of use by dilution of the stock solution to 1\times with water.

Cell cultures. We used six cancer cell lines in this study: (a) the anaplastic thyroid carcinoma cell lines ARO (25), FB1 (26), KAT4 (27), and FRO (28); (b) the 8505C cell line (29), established from an anaplastic thyroid carcinoma containing areas of papillary thyroid carcinoma; (c) the NPA cell line (28) established from a poorly differentiated thyroid carcinoma. The ARO (11), KAT4 (12), and FB1 (12) cells harbor a heterozygous BAY \textsuperscript{V600E} mutation, whereas 8505C (12), NPA, and FRO (13) express only the mutated BRAFT allele. Cells were grown in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Paisley, PA), 2 mmol/L L-glutamine, and 100 units/mL penicillin-streptomycin (Life Technologies). The P3 primary culture of normal human thyroid follicular cells was kindly donated by Francesco Curcio (Dipartimento Di Patologia Sperimentale e Clinica, Udine, Italy) and was grown as described elsewhere (30). For cell proliferation assays, 5 \times 10\textsuperscript{4} cells were plated in 35 mm dishes in 2.5% serum. The day after plating, BAY 43-9006 or vehicle was added. Cells were counted in triplicate every day. For flow cytometry analysis, 5 \times 10\textsuperscript{5} cells were plated in 100 mm dishes in 2.5% serum, and the next day they were treated with different concentrations of BAY 43-9006 or vehicle. After harvesting, cells were fixed in cold 70% ethyl alcohol in PBS. Propidium iodide (25 mg/mL) was added in the dark and samples were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett-Packard computer (Palo Alto, CA).

RNA silencing. The small inhibitor duplex RNAs targeting human BRAF were used in this study (31) were chemically synthesized by BACHMANN (Boulder, CO). Sense strands for small inhibitory duplex RNA (siRNA) targeting were as follows: BRAF, 5’-GAGAUUUGUCAUUGGAGCUCC-3’; lamin A/C, 5’-CUUGACUUCGAGAAUGCUG-3’. As a control, we used a nonspecific siRNA duplex containing the same nucleotides but in irregular sequence (scrambled). For siRNA transfection, cells were grown under standard conditions. The day before transfection, 1 \times 10\textsuperscript{6} cells were plated in 35 mm dishes in DMEM supplemented with 10% fetal bovine serum and without antibiotics. Transfection was done using 360 pmol siRNA and 18 \muL Oligofect-AMINE reagent (Invitrogen, Groningen, the Netherlands) following the instructions of the manufacturer. Cells were kept in 2.5 serum and counted 48 and 72 hours after transfection.

Protein studies. Immunoblotting experiments were done according to standard procedures. Briefly, cells were harvested in lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EGTA, 1.5 mmol/L MgCl\textsubscript{2}, 10 mmol/L NaF, 10 mmol/L sodium PPI, 1 mmol/L Na\textsubscript{2}VO\textsubscript{4}, 10 mg aprotinin/mL, and 10 mg leupeptin/mL] and clarified by centrifugation at 10,000 \times g. For protein extraction, samples of mouse xenografts were snap frozen and immediately homogenized in lysis buffer by using the Mini-Bead Beater MM300 (Qagen, Crawley, West Sussex, United Kingdom). Protein concentration was estimated with a modified Bradford assay (Bio-Rad, Munich, Germany). Antibodies were generated by an enhanced chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). Signal intensity was evaluated with the Phosphorimager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software. Anti-phospho-p44/42 MAP kinase (MAPK), specific for MAPK (ERK1/2) phosphorylated at Thr\textsuperscript{202}/Tyr\textsuperscript{204}, anti-p44/42 MAPK, anti-phospho-p90RSK (90 kDa rabinosomel S kinase), specific for p90RSK phosphorylated at Thr\textsuperscript{358}/Ser\textsuperscript{365}, anti-p90RSK, anti-phospho-MEK1/2 (MAPK1 and MAPK2), specific for MEK1 and MEK2 phosphorylated at Ser\textsuperscript{217/212}, and anti-MEK1/2 were purchased from Cell Signaling (Beverly, MA). Anti-BRAF antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-a-tubulin was from Sigma Chemical. Secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology. For the BRAF kinase assay, cells were cultured in 100 mm dishes in DMEM containing 25 mmol/L sodium PPI, 10 \muL [\textsuperscript{32P]}JATP, and 1 \muL recombinant glutathione S-transferase–MEK (Upstate Biotechnology, Inc., Lake Placid, NY). After 30-minute incubation at 4°C, reactions were stopped by adding 2 \times Laemmli buffer. Proteins were then subjected to 12% SDS gel electrophoresis. The radioactive signal was analyzed using a Phosphorimager (Molecular Dynamics, Piscataway, NJ).

Tumorogenicity in nude mice. Animals were housed in barrier facilities at the Dipartimento Di Patologia Sperimentale e Clinica, Udine, Italy. They were exposed to a 12-hour light-dark cycle and received food and water ad libitum. All manipulations were conducted in accordance with Italian regulations for experimentation on animals. No mouse showed signs of wasting or other signs of toxicity. ARO cells (1 \times 10\textsuperscript{7}/mouse) were inoculated s.c. into the right flank of 4-week-old male BALB/c nu/nu mice (The Jackson Laboratory, Bar Harbor, ME). Tumors (~ 100 mm\textsuperscript{3}) were measured with calipers. Tumor volumes (V) were
calculated by the formula: $V = \frac{A \times B^2}{2}$ (A = axial diameter; B = rotational diameter). Another group of animals (surrogate) was treated with vehicle or 60 mg/kg of BAY 43-9006 (five animals per group) for 5 days starting when the tumors reached ~300 mm$^3$. Tumors were excised 3 hours after the last dose and divided in two parts. Half of the tissue was snap-frozen in liquid nitrogen and used for protein extraction. The other half of the tissue was fixed overnight in neutral buffered formalin and processed by routine methods. Paraffin-embedded blocks were sliced into 5 µm sections and stained by HE for histologic examination or processed for immunohistochemistry. Briefly, sections were deparaffinized, alcohol-rehydrated, subjected to heat-induced antigen retrieval, and incubated overnight with anti-Ki67/MIB-1 (1:50, 3,3'-diaminobenzidine, DAKO, Carpinteria, CA) or anti-CD31 antibodies [platelet/endothelial cell adhesion molecule 1 (M-20) goat polyclonal; Santa Cruz Biotechnology; ref. 32]. Finally, the slides were incubated with biotinylated anti-IgG and with premixed avidin-biotin complex (Vectostain ABC kits, Vector Laboratories, Burlingame, CA). The immune reaction was revealed with 0.06 mmol/L diaminobenzidine (DAKO) and 2 mmol/L hydrogen peroxide. As a negative control, tissue slides were incubated with preimmune serum. Apoptotic cell death rate was assessed in tissue slides by in situ labeling of DNA strand breaks as previously described (33). Briefly, dewaxed tissue sections were digested with Proteinase K (Boehringer Mannheim, Mannheim, Germany) and processed with the in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany) used according to the instructions of the manufacturer.

Statistical analysis. Two-tailed unpaired Student’s t test (normal distributions and equal variances) were used for statistical analysis. Differences were significant when $P < 0.05$. Statistical analysis was done using the Graph Pad InStat software program (version 3.06.3, San Diego, CA).

Results

Selective knockdown of BRAF by siRNA blocks the MAPK cascade and growth of anaplastic thyroid carcinoma cell lines. We used RNA interference (RNAi) to specifically knock down BRAF expression in two anaplastic thyroid carcinoma cell lines: FRO that express only the mutated $^{V600E}$BRAF allele and ARO carrying the same mutation at the heterozygous level. We used siRNAs against BRAF and, as control, a scrambled siRNA sequence or siRNA against the housekeeping lamin A/C mRNA. Transfection with BRAF siRNA, but not with the control siRNAs, diminished BRAF, but not c-RAF, protein levels in FRO and ARO cells (Fig. 1A and C). BRAF protein knockdown was significant 48 hours after transfection (Fig. 1A and C), but only very modest after 24 hours (not shown). As a positive control of transfection, lamin A/C siRNA caused a strong inhibition of lamin protein levels (Fig. 1A and C). MEK1/2 kinases, once phosphorylated by RAF kinases at serine 217 and 221, phosphorylate threonine 202 and tyrosine 204 in the activation segment of p44 and p42 MAPK (ERK1 and 2; ref. 34). Thus, we analyzed MEK1/2 and MAPK phosphorylation upon BRAF knockdown in anaplastic thyroid carcinoma cells. Consistent with a key role of BRAF in MAPK cascade, BRAF silencing in FRO and ARO cells resulted in a reduction of p44/42 MAPK (~5-fold) and MEK1/2 (~3-fold) phosphorylation levels 48 hours after transfection (Fig. 1A and C).

We asked whether BRAF expression was required for the growth of BRAF mutation–positive anaplastic thyroid carcinoma cells. Cell counts were obtained in triplicate after FRO and ARO cell transfection with BRAF or scrambled siRNA. The transient silencing of BRAF significantly inhibited the growth of FRO and ARO cells (in 2.5% serum), whereas the negative control siRNA had virtually no effect (Fig. 1B and D). After 48 hours, FRO cells treated with scrambled RNAI numbered $77.3 \times 10^3$ and those treated with BRAF RNAI numbered $23.3 \times 10^3$ ($P < 0.0001$). After 48 hours, ARO cells treated with scrambled RNAI numbered $48 \times 10^3$ and those treated with BRAF RNAI numbered $18 \times 10^3$ ($P < 0.0001$). Thus, continued BRAF expression is essential for MAPK stimulation and growth of FRO and ARO cells.

Inhibition of oncogenic BRAF signaling in thyroid carcinoma cell lines by BAY 43-9006. Of the small-molecule RAF kinase inhibitors in clinical development, BAY 43-9006 is the furthest along (35). BAY 43-9006 is a multikinase inhibitor able to target not only RAF kinases but also receptor tyrosine kinases, including vascular endothelial growth factor receptor-2 (KDR) and platelet-derived growth factor receptor B. Thus, its anticancer activity is currently thought to be the result of the dual inhibition of RAF signaling and KDR-mediated and
platelet-derived growth factor receptor B–mediated tumor angiogenesis (23, 35).

Because BRAF expression was found to be essential for thyroid carcinoma cell growth, chemical BRAF blockade by BAY 43-9006 could exert cytostatic effects; thus, BAY 43-9006 could be exploited as a therapeutic tool for BRAF mutation–positive thyroid carcinoma models. To investigate this possibility, we studied the effects of BAY 43-9006 on the anaplastic thyroid carcinoma cell lines ARO, KAT4, and FB1, which carry the V600E BRAF mutation at the heterozygous level, and 8505C, FRO, and NPA, which carry only the mutated allele. After 12 hours of cultivation in low serum (2.5%), cells were treated for 6 hours with different concentrations of BAY 43-9006 or vehicle (NT) and the activity of MEK1/2, p44/p42 MAPK, and p90RSK (a p44/p42 MAPK substrate) was monitored by immunoblot with phosphospecific antibodies. Antibodies that recognize the same proteins also when nonphosphorylated were used for normalization. Immunoblots were examined with the Phosphorimager. Representative experiments are reported in Fig. 2. Consistent with the expression of an oncogenic BRAF, the MAPK cascade was constitutively active (even in low serum) in all the thyroid carcinomas tested. Treatment with BAY 43-9006 reduced the phosphorylation of MEK1/2, p44/p42 MAPK, and p90RSK (a p44/p42 MAPK substrate) was monitored by immunoblot with phosphospecific antibodies. Antibodies that recognize the same proteins also when nonphosphorylated were used for normalization. Immunoblots were examined with the Phosphorimager. Representative experiments are reported in Fig. 2. Consistent with the expression of an oncogenic BRAF, the MAPK cascade was constitutively active (even in low serum) in all the thyroid carcinomas tested. Treatment with BAY 43-9006 reduced the phosphorylation of MEK1/2, p44/p42 MAPK, and p90RSK with IC50 of 1 μmol/L for ARO, KAT4, and NPA cells and of 500 nmol/L for FB1, 8505C, and FRO cells. After treatment with 5 μmol/L BAY 43-9006, only residual phosphorylation levels of MEK1/2, p44/p42 MAPK, and p90RSK were detected in the carcinoma cell lines (Fig. 2).

To verify whether MAPK kinase knockdown was mediated by an inhibition of BRAF activity, we used an immunocomplex in vitro BRAF phosphorylation assay. Based on the presence of the V600E mutation, BRAF activity was high in NPA, KAT4, ARO, FRO (Fig. 3A), and FB1 and 8505C (not shown) cells, but not in normal P5 thyrocytes. We treated ARO and FRO cells with different concentrations of the compound or vehicle (NT) and examined them with the in vitro BRAF kinase assay. Figure 3B shows that BAY 43-9006 readily inhibited intrinsic BRAF enzymatic activity at the concentration of 1 μmol/L.

Fig. 2. In vivo inhibition of MAPK cascade in thyroid carcinoma cells by BAY 43-9006. The indicated thyroid carcinoma cell lines carrying homozygous or heterozygous mutations of BRAF were kept in 2.5% serum and treated with increasing concentrations of BAY 43-9006. Six hours later, cells were lysed and 50 μg of total cell lysates were analyzed by Western blotting with the indicated phosphospecific antibodies. Total amounts of MEK, MAPK, and RSK are shown for normalization. The results were quantified by the Phosphorimager. Representative of at least three different experiments.

Fig. 3. V600EBRAF kinase blockade by BAY 43-9006. A, the indicated cell lines were cultured for 12 hours in serum-deprived medium and harvested; a BRAF kinase assay was done (see below). B, cells were treated for 1 hour with different doses of BAY 43-9006 and then harvested. Cell lysates (500 μg) were immunoprecipitated with an anti-BRAF-specific antibody and subjected to a kinase assay with recombinant glutathione S-transferase– MEK (GST-MEK; 1 μg) as substrate. After 30 minutes of incubation at 4°C, reactions were stopped and proteins were subjected to 12% SDS gel electrophoresis. The radioactive signal was evaluated with the Phosphorimager. Representative of at least three different experiments.
Finally, ARO, KAT4, FRO, and NPA cells were treated (in 2.5% serum) with different concentrations of BAY 43-9006 or vehicle and counted at different time points. The average results of three independent determinations are reported in Fig. 4. BAY 43-9006 treatment readily reduced the proliferation rate of thyroid carcinoma but not of normal thyroid P5 cells. The IC50 for the four carcinoma cell lines was \(1\ \mu\text{mol/L}\); at 5 \(\mu\text{mol/L}\), BAY 43-9006 virtually arrested thyroid carcinoma cell growth \((P < 0.0001; \text{Fig. 4})\). Examination of the ARO cell cycle profile after BAY 43-9006 treatment (in 2.5% serum) by flow cytometry showed a marked G1 arrest upon treatment with 2.5 \(\mu\text{mol/L}\) BAY 43-9006. There were a few cells in the sub-G1 fraction, which indicates that BAY 43-9006 treatment results mainly in a cytostatic effect in these cells (not shown).

Inhibition of ARO-induced tumor formation in nude mice by BAY 43-9006. ARO cells were selected based on their high tumorigenic potential. Nude mice were injected with 1 \(\times\) 10^6 ARO cells and after \(\sim\) 10 days, when tumors had reached \(\sim\) 100 mm^3, animals (seven for each group) were randomized and treated orally 5 d/wk with BAY 43-9006 (30 or 60 mg/kg) or with vehicle. Tumor growth was monitored with calipers. The experiment was done twice and a representative experiment is shown in Fig. 5A. After 22 days of treatment, mice treated with BAY 43-9006 at either 30 or 60 mg/kg had significantly smaller tumors than control mice \((P < 0.0001)\). No significant improvement of the therapeutic effect was noted at 60 mg/kg with respect to 30 mg/kg, indicating that maximal therapeutic efficacy was already achieved at 30 mg/kg.

For mechanism of action studies, a group of mice \((n = 5)\) bearing tumors of \(\sim\) 300 mm^3 were treated daily with 60 mg/kg of BAY 43-9006 or with vehicle for 5 days. Three hours after the final dose, tumors were excised. Half the tissue was used for protein extraction and immunoblot analysis, and the other half was used for histologic examination. There were large areas of necrosis in tumors from treated animals at the H&E staining (Fig. 5B). Moreover, Ki67/MIB-1 immunolocalization was reduced and terminal deoxynucleotidyl transferase–mediated nick end labeling reactivity increased in treated tumors (Fig. 5B). These in vivo cell death effects were in contrast with the lack of apoptotic effects of the drug (see above) as well as of
BRAF RNAi (data not shown) in cultured cells. However, by directly targeting vascular endothelial growth factor receptors in tumor endothelium (23) and by targeting RAF-regulated vascular endothelial growth factor receptor secretion (36), BAY 43-9006 might prevent the development of tumor neovascularization and therefore cause tumor cell death. Accordingly, blood vessel counting by immunoperoxidase staining with anti-CD31 revealed that BAY 43-9006 treatment reduced the number of vessels (from 20 to 10 per microscopic field), demonstrating that BAY 43-9006 exerted antiangiogenic effects in ARO cell xenografts. To ensure that BRAF kinase inhibition in vivo participated to the therapeutic effect as well, proteins were extracted from ARO tumors treated with BAY 43-9006. As shown in Fig. 5C, tumor growth inhibition was associated with a remarkable reduction of p44/42 MAPK, MEK1/2, and, at a lower extent, RSK in vivo phosphorylation levels.

Discussion

There is an urgent need for therapies that can slow down the progression of anaplastic thyroid carcinoma. On the other hand, although papillary thyroid carcinomas in general have an excellent prognosis, there is no effective treatment for tumors that have lost radioiodine uptake. Based on the experimental and clinicopathologic evidences indicating that BRAF is involved in papillary thyroid carcinoma initiation and progression to anaplastic thyroid carcinoma, BRAF has emerged as a promising therapeutic target for thyroid carcinomas (15). By siRNA-mediated BRAF knockdown, here we could show that V600E BRAF-expressing thyroid carcinoma cells depend on continuous BRAF activity for intracellular signaling and cell proliferation; these finding suggest that indeed BRAF can be exploited to develop novel therapies for thyroid carcinomas carrying BRAF mutations.

At a preclinical level, recent insights have shown that chemically modified siRNAs can silence endogenous genes after i.v. injection in mice and, therefore, be exploited for treatment of disease (37). Moreover, injection of lentiviral vectors that produce RNAi-mediated silencing of specific genes proved efficacious in animal models of disease (38). Nevertheless, in clinical setting, molecular targeting of specific protein kinases, like ABL and KIT, with small-molecule inhibitors has already proved efficacious (39). Various BRAF inhibitors have been reported and, among them, the orally available by-aryl
urea BAY 43-9006 has reached the clinical testing stage (35). BAY 43-9006 inhibits RAF kinases and the tyrosine kinases vascular endothelial growth factor receptor 2/3, Fln-3, platelet-derived growth factor receptor B, FGFR1, and KIT (23). It inhibits the V600E BRAF mutant albeit with a slightly lesser potency than the wild-type kinase (23). BAY 43-9006 is undergoing advanced clinical trials (35). It is being tested in a phase II study of patients with locally advanced, metastatic, or recurrent thyroid cancer (www.cancer.gov/cancer/trials). Thus far, phase III is achieving promising results on renal cell carcinoma, where probably BAY 43-9006 is effective for its activity on angiogenic kinases (35). Here, we show that BAY 43-9006 targets signal transduction along the MAPK cascade and tumor cell proliferation in V600E BRAF-positive thyroid carcinoma cell lines. Although in vitro, the compound mainly exerted cytostatic effects, it also caused tumor cell death in nude mice xenografts very likely for the concomitant angiogenesis inhibition. Tumor cells are often selected to bypass the effects of antitumor agents and the simultaneous assault on both neoplastic and endothelial cells may circumvent the development of resistance. This might be an advantage of drugs like BAY 43-9006 that are able to pinpoint more than one target simultaneously (40). However, BAY 43-9006 treatment did not cause a complete regression of ARO cell tumors. Similar observations have been reported upon BAY targeting in melanomas (41).

In conclusion, these findings provide the proof-of-concept that BRAF is a therapeutic target in thyroid cancer analogous to ABL and KIT in other tumors. Thus, BAY 43-9006, and perhaps other small molecules with a similar specificity profile, holds promise for molecular therapy of thyroid cancer. In a clinical setting, it will be mandatory to know the BRAF mutational status of treated patients and to show that the compound has sufficient activity to inhibit the BRAF kinase at the concentration achieved in patient tissues. Based on the preclinical data, one possibility could be to combine the drug with other synergistic therapeutics that may facilitate tumor regression.

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References

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