BAY 43-9006 Inhibition of Oncogenic RET Mutants

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Background: **Medullary and papillary thyroid carcinomas are often associated with oncogenic activation of the RET tyrosine kinase. We evaluated whether the biaryl urea BAY 43-9006, which is known to inhibit several other tyrosine kinases, blocks RET kinase function and oncogenic activity.** ***Methods:*** **We examined BAY 43-9006 activity against oncogenic RET in vitro and in cellular RET signaling in oncogenic RET-transfected NIH3T3 fibroblasts by using immunocomplex kinase assays and immunoblotting with phospho-specific antibodies. The effects of BAY 43-9006 on proliferation of human TPC1 and TT thyroid carcinoma cells, which harbor spontaneous oncogenic RET alleles, and on RAT1 fibroblasts transformed with oncogenic RET mutants, including mutants that are resistant to other chemotherapeutic agents, were determined using growth curves and flow cytometry. Growth of TT cell–derived xenograft tumors in athymic mice treated orally with BAY 43-9006 or with vehicle was measured. All statistical tests were two-sided.** ***Results:*** **BAY 43-9006 inhibited oncogenic RET kinase activity at half-maximal inhibitory concentrations (IC_{50}s) of 50 nM or less in NIH3T3 cells. It also arrested the growth of NIH3T3 and RAT1 fibroblasts transformed by oncogenic RET and of thyroid carcinoma cells that harbor spontaneous oncogenic RET alleles. Moreover, BAY 43-9006 inhibited the growth of cells carrying RET V804L (IC_{50} = 110 nM, 95% confidence interval [CI] = 88 to 133 nM) or RET V804M (IC_{50} = 147 nM, 95% CI = 123 nM to 170 nM), both mutants that are resistant to anilinoquinazolines and pyrazolopyrimidines. After 3 weeks of oral treatment with BAY 43-9006 (60 mg/kg/day), the volume of TT cell xenografts (n = 7) was reduced from 72.5 to 44 mm^3 (difference = 28.5 mm^3, 95% CI = 7 mm^3 to 50 mm^3), whereas in vehicle-treated mice (n = 7), mean tumor volume increased to 408 mm^3 (difference = 320 mm^3, 95% CI = 180 mm^3 to 460 mm^3; untreated versus treated, P =.02). This inhibition paralleled a decrease in RET phosphorylation.** ***Conclusions:*** **BAY 43-9006 is a powerful inhibitor of the RET kinase. Its potential as a therapeutic tool for RET-positive thyroid tumors, including those expressing V804 mutations merits study.** **[J Natl Cancer Inst 2006;98:326–34]**

RET is a single-pass transmembrane tyrosine kinase receptor and is part of a cell-surface complex that binds growth factors of the glial-derived neurotrophic factor (GDNF) family in association with four different coreceptors, GFRα1–4 *(1)*. The RET gene is a potent oncogene that is involved in the pathogenesis of several human tumors. In papillary thyroid carcinoma *(2)*, chromosomal inversions or translocations cause the in-frame fusion of the tyrosine kinase-encoding domain of RET with the 5′-end of heterologous genes. The resulting RET/papillary thyroid carcinoma (PTC) chimeric sequences are oncogenic. The most frequent rearrangements are RET/PTC1 and RET/PTC3 formed by the fusion with the H4/D10S170 or the RFG/ELE1genes, respectively *(1)*. Virtually all of the translocated amino termini that have been found to be fused to RET are predicted to fold into coiled coils. These motifs provide RET/PTC kinases with the ability to undergo ligand-independent dimerization and allow constitutive activation of RET. Moreover, the promoters of the fused gene drive the expression of the rearranged RET alleles *(1)*.

Germline point mutations in RET cause the dominantly inherited multiple endocrine neoplasia (MEN) type 2A and 2B and familial medullary thyroid carcinoma. MEN 2 patients are affected by medullary thyroid carcinoma, a malignant tumor that arises from calcitonin-secreting C cells. Familial medullary thyroid carcinoma predisposes patients to medullary thyroid carcinoma alone, whereas other features can be associated with MEN 2A (pheochromocytoma, parathyroid hyperplasia, and hereditary localized pruritus) and MEN 2B (pheochromocytoma, ganglioneuromatosis of the intestine, thickening of corneal nerves, and marfanoid habitus) *(3–5)*. Most MEN 2B patients carry the M918T mutation in the RET kinase domain, and only a small fraction harbor the A883F substitution *(4,5)*. Most MEN 2A and familial medullary thyroid carcinoma patients carry mutations that affect a cysteine residue in the extracellular cysteine-rich domain of RET (most often C634). Familial medullary thyroid carcinoma is also associated with changes in the N-terminal (E768D, L790F, Y791F, V804L, and V804M) or C-terminal (S891A) regions of the RET kinase *(3–5)*. Point substitutions at V804, M918, and E768 are found in approximately 30% of patients with sporadic medullary thyroid carcinoma *(3–5)*. The mechanisms that lead to RET oncogenic conversion in MEN 2 depend on the location of the amino acid change. Extracellular cysteine mutants display constitutive kinase activity after homodimerization. Constitutive activation and altered substrate specificity have been implicated in the case of RET intracellular domain mutations *(1)*.

Although RET kinase is constitutively active in both papillary thyroid carcinoma and medullary thyroid carcinoma, the diseases are physiologically different. Local disease control by surgical resection, adjuvant radioiodine treatment, and thyroid hormone replacement are the cornerstones of treatment for papillary thyroid carcinoma *(2)*. However, if this treatment fails, patients may succumb to the disease *(6)*. Early diagnosis and

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treatment are essential for the survival of patients with medullary thyroid carcinoma, because the disease does not respond to standard chemotherapy or to conventional radiotherapy. Unfortunately, medullary thyroid carcinoma is often incurable because the cancer has metastasized to regional lymph nodes or distant sites before diagnosis. Thus, for many patients with hereditary or sporadic medullary thyroid carcinoma and for some patients with papillary thyroid carcinoma, there is no effective treatment *(6)*.

Protein kinases have become one of the most important targets for anticancer drug development. The approval of imatinib (Gleevec) for chronic myeloid leukemia and gefitinib (Iressa) and erlotinib (Tarceva) for non–small-cell lung cancer has provided proof of the principle of the effectiveness of small-molecule kinase inhibitors *(7)*. The causative role played by RET germline mutations in familial medullary thyroid carcinoma *(3–5)*, the presence of RET alterations in very early phases of papillary thyroid carcinoma and medullary thyroid carcinoma *(8)*, and the ability of RET oncogenes to initiate tumor formation in tissue-specific transgenic animals *(8)* make RET a prime target for thyroid cancer therapies. Small molecules of various chemical classes have been reported to inhibit RET; these include two pyrazolopyrimidines (PP1 and PP2) *(9–11)*, the 2-indolinone RPI-1 *(12)*, two indolocarbazole derivatives (CEP-701 and CEP-751) *(13)*, and the anilinoquinazoline ZD6474, which is in an advanced phase of clinical study *(14,15)*. A methionine or leucine substitution for valine 804 (V804M and V804L) in RET confers resistance to ZD6474, PP1, and PP2 *(16)*. V804 mutations are present alone or with other RET mutations in MEN 2 carriers (approximately 4% of patients) and in sporadic medullary thyroid carcinoma patients *(4,17–23)*. V804 in RET corresponds to residues in ABL (T315) *(24)*, epidermal growth factor receptor (EGFR) (T790) *(25,26)*, KIT (T670) *(27)*, and platelet-derived growth factor receptor A (PDGFRA) (T674) *(28)*, whose mutation mediates resistance to inhibitors of various chemical classes.

BAY 43-9006 is a biaryl urea that targets the serine/threonine kinases RAF-1 and BRAF *(29,30)* and the tyrosine kinase receptors VEGFR-2 (KDR), VEGFR-3 (Flt-4), Flt-3, PDGFR-B, and KIT *(30)*. BAY 43-9006 is an orally available cytostatic agent that is undergoing advanced clinical trials *(30)*. In this study, we exploit the ability of BAY 43-9006 to inhibit RET activity/signaling and the autonomous growth and tumorigenicity of human cell lines carrying oncogenic RET alleles.

Materials and Methods

Compounds

BAY 43-9006, *N*-(3-trifluoromethyl-4-chlorophenyl)-*N′*-(4-[2-methylcarbamoyl pyridin-4-yl]oxyphenyl) urea, was provided by Bayer HealthCare Pharmaceuticals (West Haven, CT). PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]-pyrimidine, was purchased from Alexis (San Diego, CA). For in vitro experiments, BAY 43-9006 and PP1 were dissolved in dimethyl sulfoxide. For in vivo experiments, BAY 43-9006 was dissolved in Cremophor EL–ethanol (50 : 50 Sigma Cremophor EL–95% ethyl alcohol) (Sigma Chemical Co., St. Louis, MO) at fourfold (4×) the highest dose, foil-wrapped, and stored at room temperature. The 4× stock solution was prepared fresh every 3 days. The final dosing solution was prepared on the day of use by dilution of the stock solution to 1× with water.

Immunoblotting Analysis

Protein lysates were prepared according to standard procedures. Briefly, mouse fibroblasts and human thyroid carcinoma cells or snap-frozen tumor samples were lysed in a buffer containing 50 mM HEPES (pH 7.5), 1% (vol/vol) Triton X-100, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and aprotinin at 1 μg/mL. Lysates were clarified by centrifugation at 10 000 × g for 15 minutes. Lysates containing comparable amounts of proteins, as estimated by a modified Bradford assay (Bio-Rad, Munich, Germany) *(31)*, were subjected to direct Western blotting. Immune complexes were detected with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfort, UK). Signal intensity was analyzed using a PhosphorImager (Typhoon 8600, Amersham Pharmacia Biotech) interfaced with the ImageQuant software. Anti-phospho-Shc (1 : 1000), which recognizes phosphorylated Shc at Y317, was a rabbit polyclonal antibody from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Shc (1 : 1000) was a rabbit polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mitogen-activated protein kinase (MAPK) (1 : 1000) and anti-phospho-MAPK (1 : 1000), which recognizes p44/42MAPK (ERK1/2) phosphorylated at Thr202/Tyr204, were rabbit polyclonal antibodies from Cell Signaling (Beverly, MA). Anti-RET (1 : 1000) is a rabbit polyclonal antibody raised against the tyrosine kinase protein fragment of human RET *(32)*. Anti-phospho905 is a phospho-specific polyclonal antibody that recognizes RET proteins that are phosphorylated at Y905 *(10)*. Blots were incubated with primary antibodies for 1 hour at room temperature, followed by three washes in buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20). The blots were then incubated with the goat anti-rabbit secondary antibody (1 : 5000) coupled to horseradish peroxidase (Santa Cruz Biotechnology) for 1 hour at room temperature followed by three washes in buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20). Each experiment was performed at least three times.

Cell Culture

Parental murine NIH3T3 fibroblasts and NIH3T3 cells stably transfected with the RET mutants RET/PTC3, RET/C634R (MEN 2A), and RET/M918T (MEN 2B), the EGFR/RET chimeric receptor (the extracellular and transmembrane portions of the EGFR fused to the intracellular domain of RET), and GFRα1 (GDNF receptor α1) plus wild-type RET are described elsewhere *(14,32)*. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum, 2 mM L-glutamine, and penicillin-streptomycin at 100 units/mL (GIBCO, Paisley, PA). Epidermal growth factor (EGF) was purchased from Upstate Ltd. (Charlottesville, VA); GDNF was purchased from Alomone Labs (Jerusalem, Israel). The TPC1 cell line, derived from a papillary thyroid carcinoma harboring the RET/PTC1 rearrangement *(33)*, was cultured in DMEM with 10% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin at 100 units/mL. The TT cell line, derived from a medullary thyroid carcinoma (MTC) harboring the RET/C634W

mutation *(34)*, was cultured in RPMI-1640 with 20% fetal calf serum, 2 mM L-glutamine, and 100 units/mL penicillin-streptomycin (GIBCO). Parental Fischer rat–derived RAT1 fibroblasts and RAT1 transformed by RET/C634R, RET/V804L, or RET/V804M are described elsewhere *(35)* and were cultured in DMEM with 10% fetal calf serum, 2 mM L-glutamine, and 100 units/mL penicillin-streptomycin (GIBCO). All RET constructs used in this study encoded the short isoform of the RET protein (RET-9) *(1)*.

In Vitro Kinase Assays

For the in vitro RET autophosphorylation assay, subconfluent NIH3T3 cells stably transfected with RET/PTC3 were solubilized in lysis buffer without phosphatase inhibitors (sodium fluoride, sodium pyrophosphate, and sodium vanadate). Then, 200 μg of proteins were immunoprecipitated with anti-RET; immunocomplexes were recovered with protein A–Sepharose beads, washed five times with kinase buffer (20 mM HEPES at pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 15 mM $MnCl_2$, and 15 mM $MgCl_2$) and incubated 20 minutes at room temperature in kinase buffer containing 2.5 μCi of [γ-^{32}P]ATP and unlabeled ATP (20 μM) *(9)*. Samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were dried and exposed to film for autoradiography. Signal intensity was analyzed using a PhosphorImager (Typhoon 8600) interfaced with the ImageQuant software. For phosphorylation of the synthetic substrate, RET immunocomplexes were incubated (20 minutes at room temperature) in kinase buffer containing 200 μM poly-(L-glutamic acid-L-tyrosine [poly-GT]) (Sigma), 2.5 μCi of [γ-^{32}P]ATP, and unlabeled ATP (20 μM). Samples were spotted on Whatman 3MM paper (Springfield Mill, UK), and ^{32}P incorporation was measured with a beta counter scintillator (Beckman Coulter, Unterschleissheim-Lohhof, Germany). Each experiment was performed at least three times.

Growth Curves and Cell Cycle Analysis

NIH3T3 (10 000/dish) and RAT1 fibroblasts (10 000/dish) and human thyroid carcinoma TPC1 (35 000/dish) and TT (90 000/dish) cells were seeded in 60-mm dishes. Fibroblasts were maintained in medium supplemented with 1% calf (NIH3T3) or fetal calf (RAT1) serum. Human cells were maintained in 2% (TPC1) or 10% (TT) fetal calf serum. The next day, BAY 43-9006 or vehicle was added to the medium and changed every 2 days. Cells were counted every 2 (fibroblasts) or 2–3 (human cell lines) days. For flow cytometry analysis, cells were grown to subconfluence in 100-mm dishes and then treated with vehicle or 1.0 μM BAY 43-9006 for 24 hours. After harvesting, cells were fixed in cold 70% ethanol in phosphate-buffered saline. Cells were washed and resuspended in phosphate-buffered saline. Propidium iodide (25 μg/mL) was added, and samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett Packard computer (Palo Alto, CA). Experiments were performed three times in duplicate.

Tumor Growth in Athymic Mice

Mice (n = 14) were housed in barrier facilities that provided 12-hour light-dark cycles and received food and water ad libitum at the Dipartimento di Biologia e Patologia Cellulare e Molecolare (University of Naples "Federico II," Naples, Italy). This study was conducted in accordance with Italian regulations for experimentation on animals. All manipulations were performed while mice were under isoflurane gas anesthesia. No mouse showed signs of wasting or other signs of toxicity. TT cells (1×10^7/mouse) were inoculated subcutaneously into the right dorsal portion of 4-week-old male BALB/c *nu/nu* mice (The Jackson Laboratory, Bar Harbor, ME). When tumors measured ~70 mm^3, after approximately 30 days, mice were randomized to receive BAY 43-9006 (n = 7, 60 mg/kg/day) or vehicle (n = 7, Cremophor EL–ethanol) alone by oral gavage for 5 consecutive days/week for 3 weeks. Tumor diameters were measured at regular intervals with calipers. Tumor volumes (V) were calculated with the formula: $V = A \times B^2/2$ (A = axial diameter; B = rotational diameter). Mice were killed by cervical dislocation, and tumors were excised and divided in two parts. Half of the tissue was snap-frozen in liquid nitrogen and used for protein extraction. The other half was fixed overnight in neutral

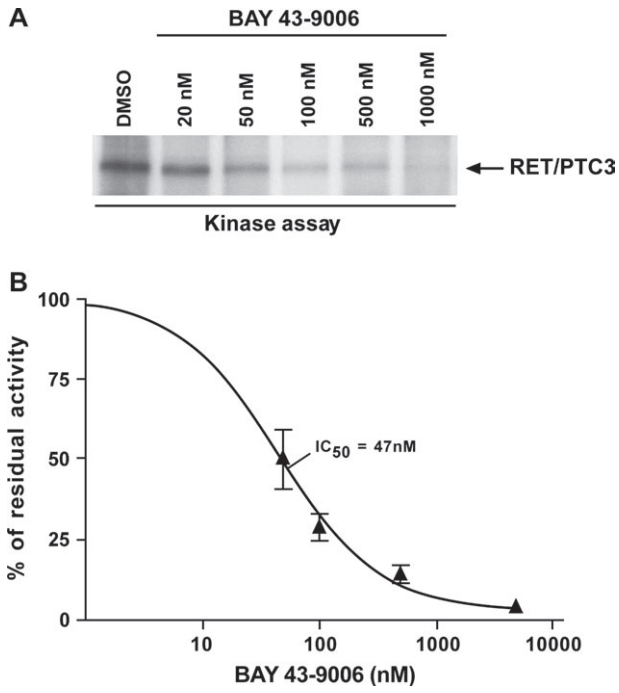

Fig. 1. In vitro inhibition of RET/papillary thyroid carcinoma (PTC) 3 by BAY 43-9006. **A**) In vitro RET autophosphorylation assay. Protein extracts from NIH-RET/PTC3 cells were immunoprecipitated with rabbit polyclonal anti-RET antibody and subjected to an immunocomplex kinase assay in the presence of [γ-^{32}P]ATP. BAY 43-9006 or vehicle alone (dimethyl sulfoxide [DMSO]) was added to the reaction mixture to reach the indicated concentrations. Reaction products were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and phosphorylated proteins were detected by autoradiography and quantified using a PhosphorImager. A representative blot from three independent experiments is shown. Aliquots of the immunoprecipitates were subjected to anti-RET Western blot for normalization (data not shown). **B**) In vitro poly-GT phosphorylation assay. Protein extracts from NIH-RET/PTC3 cells were immunoprecipitated with the anti-RET antibody and subjected to a kinase assay with poly-(L-glutamic acid-L-tyrosine (poly-GT) as a synthetic substrate in the presence of [γ-^{32}P]ATP and different concentrations of vehicle or BAY 43-9006. The phosphorylated poly-GT was spotted on filter paper, and radioactivity was counted by scintillation. The results are reported as residual poly-GT phosphorylation levels compared with the control (DMSO). The concentration of drug that inhibited activity by 50% (IC_{50}) is shown. Each **point** represents the mean value from four independent determinations; **error bars** represent 95% confidence intervals.

buffered formalin and processed by routine methods. Paraffin-embedded blocks were sliced into 5-μm sections and stained by hematoxylin and eosin for histologic examination or processed for immunohistochemistry.

Statistical Analysis

Kinase activity curves were graphed using the curve-fitting PRISM software (GraphPad Software). To compare cell growth we used the unpaired Student's *t* test (normal distributions and equal variances) and the JMP software program (version 5.1.1; SAS Institute, Inc, Austin, TX). To compare tumor growth we used the paired Student's *t* test (normal distributions and equal variances) and the JMP software program (version 5.1.1; SAS Institute), an analysis of variance (linear mixed-effect model) test for repeated measures and the Wilcoxon's rank-sum test and the Instat software program (GraphPad Software). All *P* values were two-sided, and differences were statistically significant at $P<.02$.

Results

BAY 43-9006 Effects on Oncogenic RET Autophosphorylation In Vitro

Oncogenic RET proteins undergo autophosphorylation in vitro in the absence of ligand *(32)*. We used an in vitro autophosphorylation assay to determine whether BAY 43-9006 inhibited the autophosphorylation of RET/PTC3 (i.e., oncogenic variant) kinase immunopurified from stably transfected NIH3T3 cells. BAY 43-9006 inhibited RET/PTC3 autophosphorylation with an IC_{50} of roughly 50 nM (Fig 1, A). We performed a second in vitro enzymatic assay to measure the ability of RET/PTC3 to phosphorylate a synthetic poly-GT substrate. BAY 43-9006 blocked this activity of RET/PTC3 with an IC_{50} of 47 nM (95% CI = 34 nM to 61 nM) (Fig. 1, B).

Inhibition of RET Signaling and Cell Proliferation in RET-Transformed Cells by BAY 43-9006

We next determined whether BAY 43-9006 could also inhibit the kinase activity of oncogenic RET mutants in intact cells. We treated NIH3T3 fibroblasts expressing one of three oncogenic versions of RET (RET/PTC3, RET/C634R, or RET/M918T) with BAY 43-9006 for 2 hours. We then measured RET phosphorylation levels by immunoblotting with an antibody that recognizes RET only when it is phosphorylated at tyrosine 905 (Y905) *(10,36)*. Treatment with BAY 43-9006 reduced the phosphotyrosine content of RET/PTC3, RET/C634R, and RET/M918T with an IC_{50} of 20–50 nM (Fig. 2, B–D). The three RET kinases were almost completely inhibited by 100 nM BAY 43-9006 (Fig. 2, B–D). We used two cell systems to test whether BAY 43-9006 could also inhibit wild-type RET: NIH3T3 fibroblasts that express the EGFR/RET chimera (in which the RET kinase can be stimulated by EGF) and those that coexpress wild-type RET and GFRα1 (in which the RET kinase can be stimulated by GDNF) (Fig. 2, A). BAY 43-9006 inhibited autophosphorylation of both EGFR/RET and wild-type RET (Fig. 2, E–F).

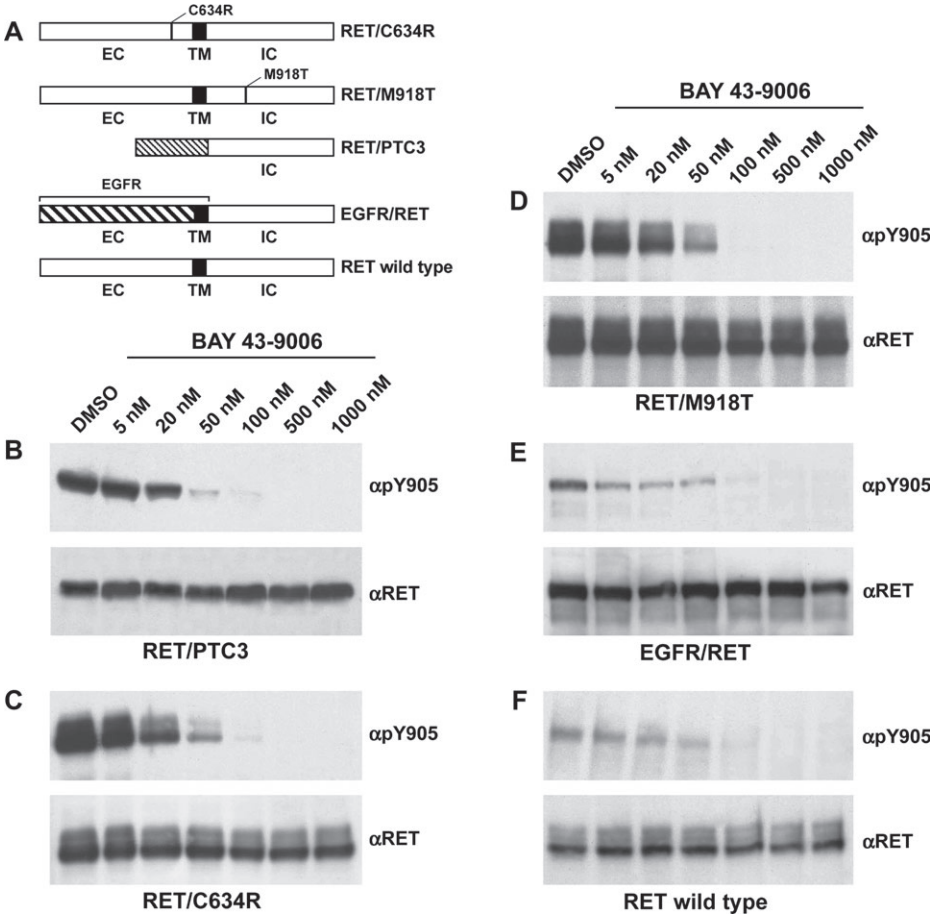

Fig. 2. In vivo inhibition of phosphorylation of wild-type RET and of RET/papillary thyroid carcinoma (PTC) 3, RET/C634R, RET/M918T, and epidermal growth factor receptor (EGFR)/RET by BAY 43-9006 in transfected NIH 3T3 cells. **A)** Schematic representation of the various constructs. EC = extracellular domain; IC = intracellular domain; TM = transmembrane domain. **B–F**) Serum-starved cells (24 hours) were treated with vehicle (dimethyl sulfoxide [DMSO]) or different concentrations of BAY 43-9006 for 2 hours; before harvesting, EGFR/RET and glial-derived neurotrophic factor (GDNF) family receptor α1 (GFRα1) + RET expressing cells were treated for 10 minutes with 100 ng/mL of epidermal growth factor (EGF) or GDNF, respectively. Cell lysates (50 μg) were immunoblotted with a rabbit polyclonal anti-phospho-RET/Y905 (αpY905) antibody to detect phosphorylation and with anti-RET (αRET) as a control for protein loading and transfer. The signal was quantified using a PhosphorImager. Representative blots from three independent experiments are shown.

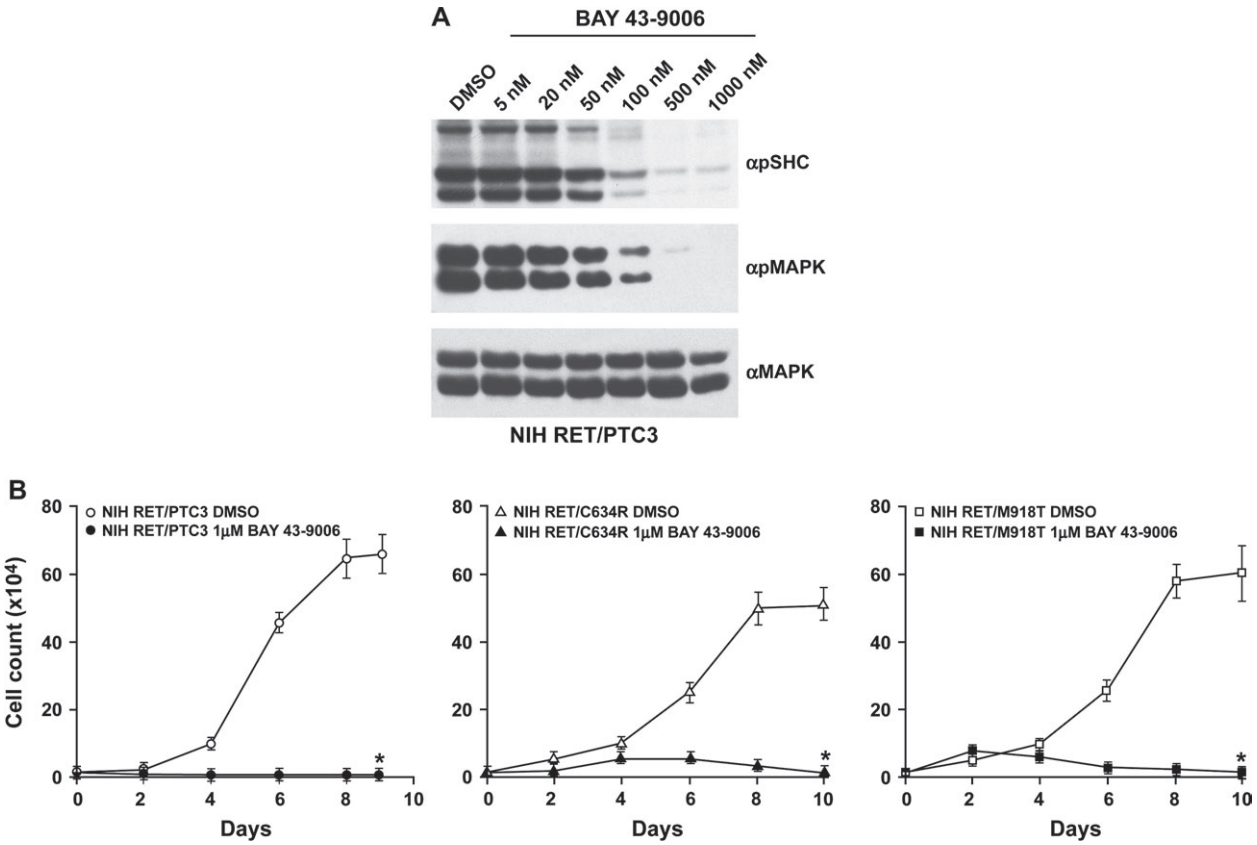

Fig. 3. Inhibition of RET-mediated growth and signaling by BAY 43-9006. **A**) NIH3T3 cells transfected with RET/papillary thyroid carcinoma (PTC) 3 were serum-starved for 24 hours and then treated with vehicle (dimethyl sulfoxide [DMSO]) or increasing concentrations of BAY 43-9006. Cell lysates (50 μg) were immunoblotted with rabbit polyclonal anti-phospho-specific Shc or p44/42 mitogen-activated protein kinase (MAPK) antibodies and with anti-MAPK antibody as a control for protein loading and transfer. The signal was analyzed using a PhosphorImager. A representative blot from three independent experiments is shown. **B**) The indicated cell lines were incubated with DMSO or 1.0 μM BAY 43-9006 in 1% calf serum, and the cells were counted at different time points. Each **point** represents the mean value for five dishes, and **error bars** represent 95% confidence intervals. *P* values were determined by the two-tailed unpaired Student's *t* test. **P*<.001.

Constitutively active oncogenic versions of RET activate the RAS/RAF/MAPK pathway by recruiting Grb2/Sos complexes through the Shc protein *(1,37)*. Accordingly, we treated RET/PTC3 cells with increasing concentrations of BAY 43-9006 and analyzed Shc and p44/p42MAPK phosphorylation by immunoblotting with phospho-specific antibodies. BAY 43-9006 inhibited RET/PTC3-dependent phosphorylation of Shc and p44/42MAPK with an IC_{50} of approximately 50 nM (Fig. 3, A). Similar results were obtained with RET/C634R and RET/M918T mutants (not shown).

We studied the effects exerted by BAY 43-9006 on the growth of NIH3T3 cells transformed by RET/PTC3, RET/C634R, and RET/M918T that were grown in low serum (2.5%) for 10 days. Proliferation of NIH3T3 cells transformed with any of these RET mutants was virtually arrested after treatment with 1 μM of BAY 43-9006 (Fig. 3, B). Fewer RET/PTC3 cells remained after treatment with 1 μM of BAY 43-9006 than after treatment with vehicle (8.4×10^3, 95% CI = 7.2×10^3 to 9.6×10^3 versus 730.5×10^3, 95% CI = 684×10^3 to 776×10^3; $P<.001$). Results were similar for RET/C634R and RET/M918T cells after treatment with 1 μM of BAY 43-9006 or with vehicle (RET/C634R cells, 8.1×10^3, 95% CI = 6.7×10^3 to 9.6×10^3, versus 552×10^3, 95% CI = 509×10^3 to 594×10^3; $P<.001$; and RET/M918T cells, 11×10^3, 95% CI = 7.9×10^3 to 14×10^3, versus 612 x 10^3, 95% CI = 591×10^3 to 634×10^3 $P<.001$). Hence, BAY 43-9006 antagonized RET oncogenic activity by blocking its kinase function and its signaling and mitogenic effects.

Effects of BAY 43-9006 on Human Carcinoma Cells Harboring a Constitutively Active RET Oncogene

We next investigated the effects of BAY 43-9006 on the TPC1 cell line, which is derived from a human PTC bearing the RET/PTC1 rearrangement *(33)*, and the TT cell line, which is derived from a human MTC harboring the RET/C634W mutation *(34)*. Treatment of either cell line with 100 nM BAY 43-9006 almost completely abrogated RET and Shc phosphorylation (Fig. 4, A and B). This treatment abrogated p44/p42 MAPK phosphorylation in TT cells and strongly reduced it (by approximately 50%) in TPC1 cells (Fig. 4, A and B).

We next measured the growth rates of TPC1 (grown in 2% serum) and TT (grown in 10% serum) cells treated with three concentrations of BAY 43-9006 (Fig. 4, C). Fewer TPC1 cells remained after treatment for 6 days with 1000 nM BAY 43-9006 than with vehicle (21×10^3, 95% CI = 17×10^3 to 24×10^3, versus 135×10^3, 95% CI = 127×10^3 to 143×10^3; $P<.001$). The number of TPC1 cells remaining after 6 days of treatment with 250 nM BAY 43-9006 was lower than that of cells treated with vehicle (65×10^3, 95% CI = 59×10^3 to 71×10^3, versus 135×10^3, 95% CI = 127×10^3 to 143×10^3; $P<.001$). A reduction of TPC1 growth was still observed at a 100 nM dose (116×10^3, 95% CI = 107×10^3 to 125×10^3, versus 135×10^3, 95% CI = 127×10^3 to 143×10^3; $P<.001$). Fewer TT cells remained after 10 days of treatment with 1000 nM BAY 43-9006 than with vehicle

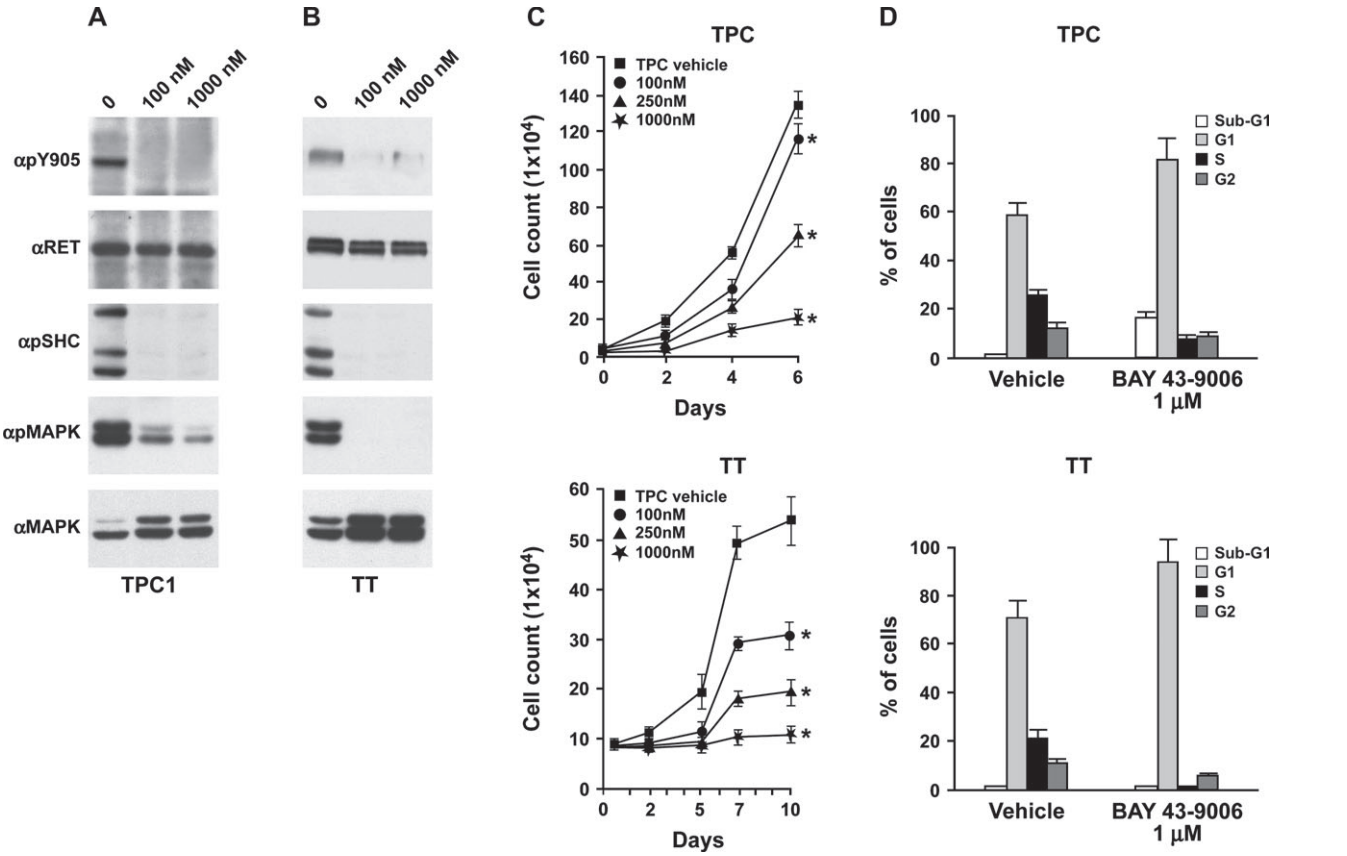

Fig. 4. Inhibition of RET-mediated growth and signaling by BAY 43-9006 in human cells. TPC1 (**A**) and TT (**B**) cell lines were serum-starved for 24 hours and then treated with vehicle or BAY 43-9006. Cell lysates (50 μg) were immunoblotted with rabbit polyclonal anti-phospho-RET, phospho-Shc, phospho-mitogen-activated protein kinase (MAPK), and MAPK antibodies as a control for protein loading and transfer. Representative blots from three independent experiments are shown. **C**) TPC1 and TT cells were incubated with vehicle, 100, 250, or 1000 nM BAY 43-9006 in 10% and 2% serum, respectively, and counted at different time points. Each **point** represents the mean value for five dishes and **error bars** represent 95% confidence intervals. *P* values were determined using the two-tailed unpaired Student's *t* test. **P*<.002. **D**) After 24 hours of serum starvation, TPC1 and TT cells were treated with vehicle or with 1.0 μM BAY 43-9006 for 24 hours and subjected to flow cytometry. The percentages of cells in the sub-G_1 (apoptotic), G_0/G_1, S, and G_2/M compartments are indicated. Means of three independent experiments, each performed in duplicate, and 95% confidence intervals are shown.

(109×10^3, 95% CI = 100×10^3 to 118×10^3, versus 541×10^3, 95% CI = 487×10^3 to 584×10^3; $P<.001$). The number of TT cells remaining after 10 days of treatment with 250 nM BAY 43-9006 was lower than that after treatment with vehicle (199×10^3, 95% CI = 187×10^3 to 211×10^3, versus 541×10^3, 95% CI = 487×10^3 to 584×10^3; $P<.001$). We also observed growth inhibition at 100 nM BAY 43-9006 (309×10^3, 95% CI = 285×10^3 to 332×10^3, versus 541×10^3, 95% CI = 487×10^3 to 584×10^3; $P<.001$).

Examination of the TT and TPC1 cell cycle profiles by flow cytometry showed a marked G_1 arrest of both cell lines upon treatment with 1 μM BAY 43-9006 (Fig. 4, D). There were approximately 10-fold more TPC1 cells in the sub-G_1 fraction after BAY 43-9006 treatment compared with vehicle treatment. In addition to its cytostatic effect, BAY 43-9006 exerts a proapoptotic effect at this drug concentration. Thus, BAY 43-9006 blocks oncogenic RET signaling in TT and TPC1 cells and has a mainly cytostatic effect.

Inhibition of RET/V804 Mutants by BAY 43-9006

Mutations of valine 804 in RET to leucine (V804L) or methionine (V804M) (Fig. 5, A) render RET resistant (approximately 50-fold increase of the IC_{50}) to the small-molecule tyrosine kinase/RET inhibitors PP1, PP2, and ZD6474 *(16)*. We measured the effect of BAY 43-9006 on the activity of RET/V804L and RET/V804M kinases using the in vitro poly-GT kinase assay. Despite their resistance to other inhibitors, both mutants were only two- to threefold less sensitive than RET/C634R to inhibition by BAY 43-9006. The IC_{50} of BAY 43-9006 was 110 nM for RET/V804L (95% CI = 88 nM to 133 nM) and 147 nM for RET/V804M (95% CI = 123 nM to 170 nM), whereas the IC_{50} of BAY 43-9006 for RET/C634R was 49 nM (95% CI = 35 nM to 62 nM) (Fig. 5, B).

We sought to verify these findings in intact cells. RAT1 fibroblasts expressing the RET/V804L or the RET/V804M alleles were treated for 2 hours with vehicle, BAY 43-9006, or PP1 (500 or 1000 nM), and RET phosphorylation was measured by immunoblotting. Similar to the in vitro data, only residual phosphorylation of the two mutant proteins (more pronounced for V804M) was detected after treatment with 500 nM BAY 43-9006 (Fig. 5, C). Mutant RET phosphorylation was virtually abrogated by 1000 nM BAY 43-9006 (Fig. 5, C). As previously reported *(16)*, PP1 only slightly inhibited RET phosphorylation at these doses (Fig. 5, C).

We studied the effects exerted by BAY 43-9006 on the growth rate of RAT1 cells transformed by RET/V804M and RET/C634R (Fig. 5, D). Fewer RET/C634R cells remained after 9 days of treatment with 0.1 μM BAY 43-9006 than with vehicle (46×10^4,

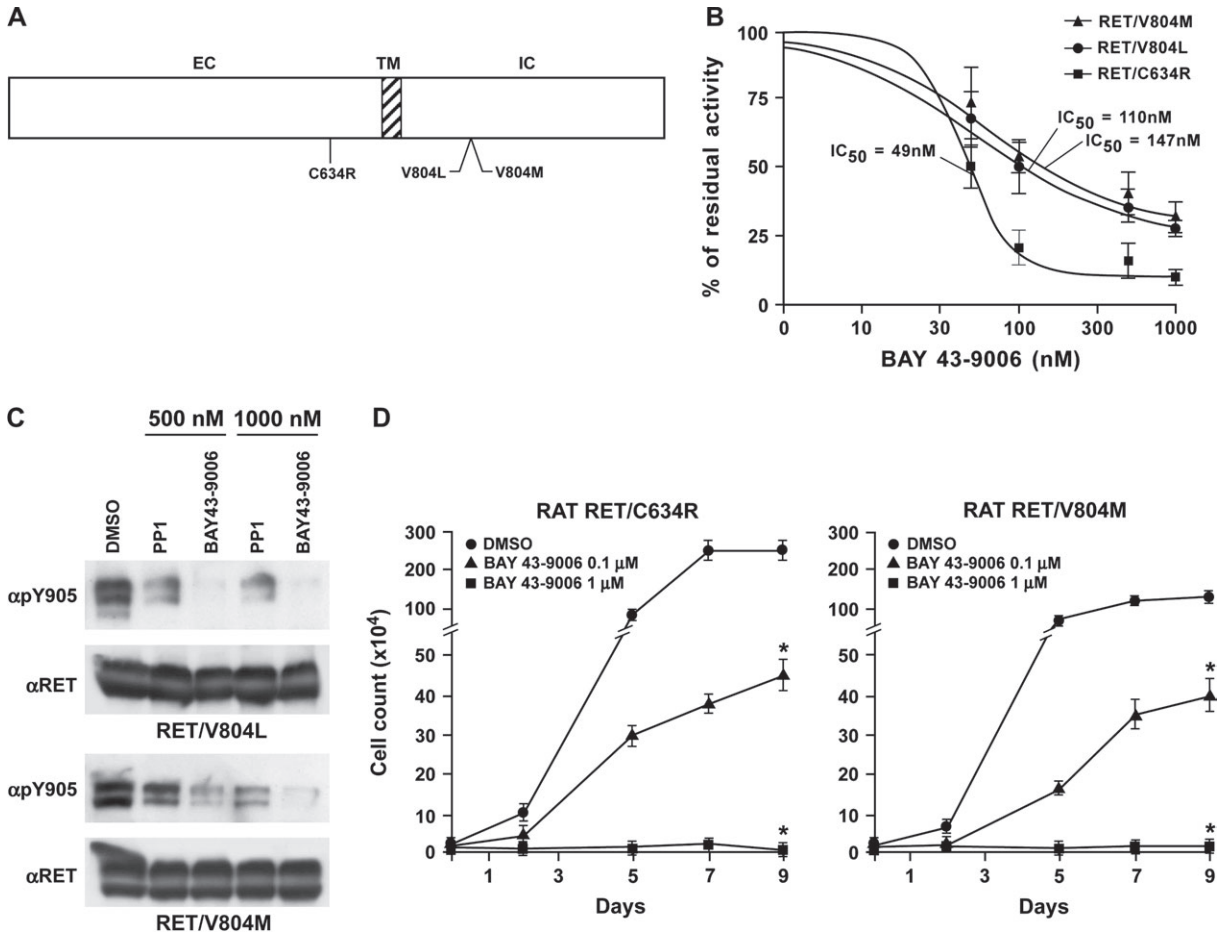

Fig. 5. Inhibition of RET mutants in transformed RAT1 cells by BAY 43-9006. **A**) Schematic representation of RET/V804L, RET/V804M, and RET/C634R mutants. EC = extracellular domain; IC = intracellular domain; TM = transmembrane domain. **B**) In vitro poly-(L-glutamic acid-L-tyrosine (poly-GT) phosphorylation assay. Proteins from RAT1 cells expressing the indicated constructs were immunoprecipitated with rabbit polyclonal anti-RET antibody and subjected to the poly-GT kinase assay. The means of results from four independent experiments were averaged and reported as residual poly-GT phosphorylation levels compared with the control (dimethyl sulfoxide [DMSO]). **C**) Protein extracts from RAT1 cells expressing the indicated constructs and treated for 2 hours with DMSO, BAY 43-9006, or PP1 were immunoblotted with rabbit polyclonal anti-phospho-RET or anti-RET antibodies. **D**) RAT1 cells expressing the indicated constructs were incubated with DMSO, BAY 43-9006, or PP1 in 1% serum and counted at different time points. Each **point** represents the mean value of five replicates and **error bars** represent 95% confidence intervals. *P* values were determined using the two-tailed unpaired Student's *t* test. **P*<.001.

95% CI = 40×10^4 to 52×10^4, versus 261×10^4, 95% CI = 222.5×10^4 to 300×10^4; $P<.001$). Similarly, fewer RET/V804M cells remained after 9 days of treatment with 0.1 μM BAY 43-9006 than with vehicle (40.2×10^4, 95% CI = 38×10^4 to 42.5×10^4 versus 133×10^4, 95% CI = 124×10^4 to 142×10^4; $P<.001$). The proliferation of RAT1 fibroblasts expressing either RET/C634R or RET/V804M was virtually abrogated after treatment with 1 μM BAY 43-9006 (Fig. 5, D).

Inhibition of TT-Induced Tumor Growth in Nude Mice by BAY 43-9006

To investigate the effects of BAY 43-9006 on medullary thyroid carcinoma tumor growth, we injected nude mice (subcutaneous, right dorsal) with 1×10^7 TT cells. After approximately 30 days, when tumors measured approximately 80 mm^3, mice (seven in each group) were randomized to receive BAY 43-9006 (60 mg/kg/day) or vehicle 5 days/week for 3 weeks. Treatment with BAY 43-9006 strongly reduced tumor growth (Fig. 6). After 21 days, the mean volume of tumors in mice treated with BAY 43-9006 decreased (from 72.5 to 44 mm^3, difference = 28.5 mm^3, 95% CI = 7 mm^3 to 50 mm^3; $P = .018$), whereas that of mice treated with vehicle increased (from 87 to 408 mm^3, difference = 320 mm^3, 95% CI = 180 mm^3 to 460 mm^3; $P<.001$) (Fig. 6, A). Analysis of variance (linear mixed-effect model) test for repeated measures and the Wilcoxon rank-sum test demonstrated that differences between treated and untreated animal were statistically significant ($P<.001$ and $P = .02$, respectively). Treated tumors showed a cytoreduction, probably because of the extensive necrosis occurring upon treatment (Fig. 6, B). Ki67/MIB-1 immunostaining was reduced in treated tumors, which is consistent with a reduced mitotic index (not shown). More important, we observed a strong reduction of in vivo RET phosphorylation in proteins that were extracted from tumors in BAY 43-9006-treated versus vehicle-treated mice (Fig. 6, C).

Discussion

Here, we have shown that BAY 43-9006 inhibits RET enzymatic function. It inhibited RET signaling and growth of RET-transfected fibroblasts and human thyroid cancer cells that harbor RET/PTC and RET/MEN 2 oncogenes. Furthermore, BAY 43-9006 blocked growth of xenograft tumors that were derived from a MTC cell line.

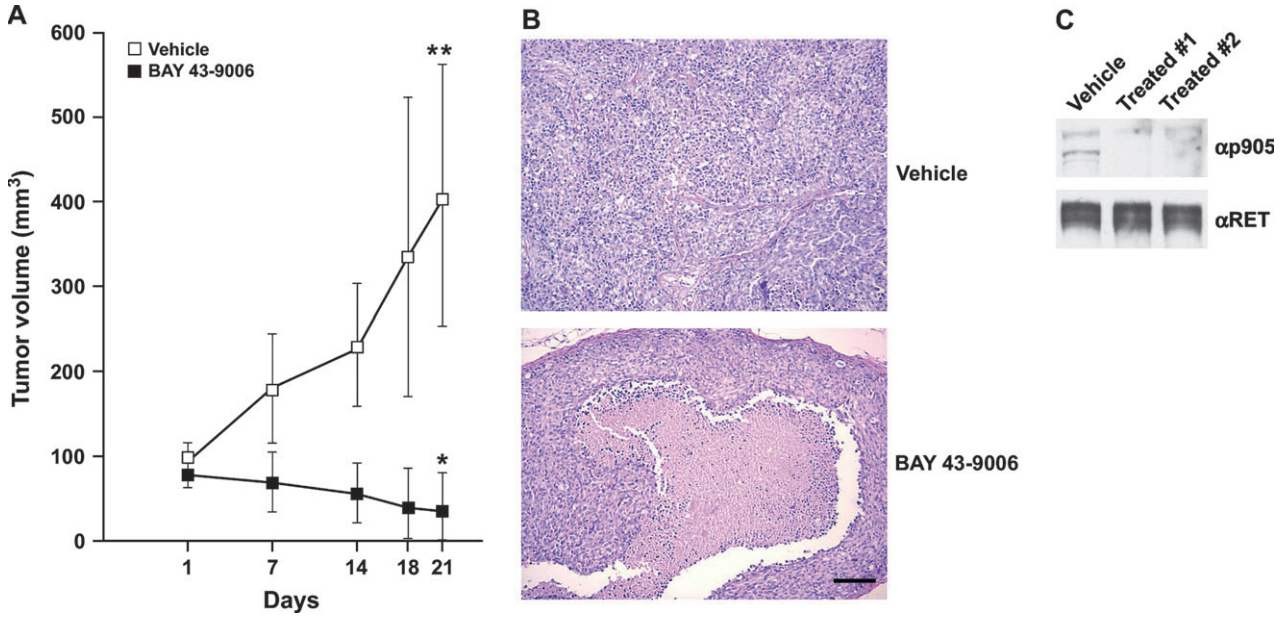

Fig. 6. Anti-tumorigenic effects of BAY 43-9006 in TT cell xenografts. **A**) TT cells (1×10^7/mouse) were injected subcutaneously into the right dorsal portion of BALB/c athymic mice. When tumors measured approximately 80 mm^3, mice were randomized to two groups (7 mice/group) to receive BAY 43-9006 (60 mg/kg/day) or vehicle (Cremophor EL–ethanol) by oral gavage. Treatment was administered for 5 consecutive days/week for 3 weeks (day 1 is the treatment starting day). Tumor diameters were measured with calipers, and tumor volumes were calculated. **Error bars** represent 95% confidence intervals. *P* values (two-sided) were determined by analysis of variance (linear mixed-effect model) for repeated measures and paired Student's *t* test for tumor changes within the treated (*$P = .018$) or untreated group (**, $P < .001$). **B**) Tumors were excised and examined by conventional hematoxylin and eosin staining. Representative micrographs are shown. **Bar** = 1 mm. **C**) Proteins (1000 μg) extracted from two representative tumors (on day 21) from untreated and treated mice were immunoprecipitated with rabbit polyclonal anti-RET antibody and immunoblotted with either anti-pY905 or anti-RET.

BAY 43-9006 is a biaryl urea that targets the RAF family serine/threonine kinases RAF-1 and BRAF *(29,30)* and the tyrosine kinase receptors VEGFR2 (KDR), VEGFR3 (Flt-4), Flt3, PDGFRB, and KIT *(30)*. BAY 43-9006 probably inhibits the growth of RET-driven tumors through a combination of these activities and targets both VEGF-dependent tumor angiogenesis and RET-dependent thyroid cancer cell proliferation. Intriguingly, the anilinoquinazoline ZD6474 also exerts both anti-RET *(14)* and anti-VEGFR activities *(38)*.

Molecular resistance is the major obstacle to targeted cancer therapy with small-molecule kinase inhibitors *(24)*. For example, relapses after an initial response are frequent in chronic myelogenous leukemia due to the emergence of cells that are resistant to imatinib *(39,40)*. This resistance is primarily mediated by mutations that either 1) allosterically prevent the ABL kinase from adopting the inactive conformation to which imatinib binds or 2) directly target the imatinib binding site. An example of the second type of mutation is the threonine-to-isoleucine substitution at position 315 in ABL (T315I) *(39–42)*. Consequently, threonine 315 in ABL and the homologous residues in other receptor tyrosine kinases (threonine 790 in EGFR, threonine 674 in PDGFRA, and threonine 670 in KIT) have been designated "gatekeepers," because their mutation causes resistance to various small-molecule inhibitors *(25–28)*. The homologous residue in RET is V804, which is a determinant of susceptibility to pyrazolopyrimidines and anilinoquinazolines *(16)*. Here we show that V804L and V804M only slightly (a two- and threefold increase in IC_{50}, respectively) affect RET susceptibility to BAY 43-9006. These findings also raise the possibility that BAY 43-9006 might be of benefit in patients who harbor RET mutations at V804 [rare MEN 2 families and some sporadic medullary thyroid carcinoma patients *(4–5,17–23)*], who thus might have a "primary" resistance to other inhibitors. Structural analysis of BAY 43-9006 binding to the RET kinase would give insight as how to design inhibitors that can overcome drug resistance toward gate-keeper mutants.

The study has several potential limitations. Given the lack of a V804 mutation-positive MTC cell line, we could not verify the in vivo activity of BAY 43-9006 on this oncogenic form of RET. Also we cannot exclude the possibility that RET mutants, other than those tested in this study, may have resistance to the compound.

In conclusion, we have shown that BAY 43-9006 targets RET-derived oncoproteins and blocks the growth of MTC xenografts. Moreover we have shown the efficacy of the compound on V804-resistant mutants. The preclinical findings reported here suggest that BAY 43-9006 might offer a potential treatment strategy for papillary and medullary thyroid carcinomas sustaining oncogenic activation of RET. Nevertheless, only by testing the activity of the compound in thyroid cancer patients will it be possible to assess the clinical value of RET inhibition by BAY 43-9006.

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Notes

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