Sorafenib inhibits the angiogenesis and growth of orthotopic anaplastic thyroid carcinoma xenografts in nude mice

Seungwon Kim, Yasemin D. Yazici, Gabriel Calzada, Maher N. Younes, Samar A. Jasser, Adel K. El-Naggar, and Jeffrey N. Myers

Departments of 1Head and Neck Surgery, 2Pathology, and 3Cancer Biology, The University of Texas M. D. Anderson Cancer Center and 4Department of Otolaryngology-Head and Neck Surgery, Baylor College of Medicine, Houston, Texas

Abstract

Anaplastic thyroid carcinoma (ATC) remains one of the most lethal human cancers. We hypothesized that sorafenib, a multikinase inhibitor of the BRAf, vascular endothelial growth factor receptor-2, and platelet-derived growth factor receptor-β kinase, would decrease tumor growth and angiogenesis in an orthotopic model of ATC. The in vitro antiproliferative and proapoptotic effects of sorafenib on ATC cell lines were examined. To study the in vivo effects of sorafenib on orthotopic ATC tumors in nude mice, sorafenib was given p.o. at 40 or 80 mg/kg daily. Intratumoral effects were studied using immunohistochemical analysis. The effect of sorafenib on survival of the mice was also studied. Sorafenib inhibited the in vitro proliferation of ATC cell lines. Sorafenib also significantly inhibited tumor angiogenesis via the induction of endothelial apoptosis in an orthotopic model of thyroid cancer. As result, the growth of orthotopic ATC xenografts was reduced and the survival of the test animals was improved. Sorafenib exerts significant antitumor activity in an orthotopic xenograft model of ATC via a potent antiangiogenic effect. The antiangiogenic effects of sorafenib suggest that its use in clinical setting may not depend on the BRAF mutational status of thyroid tumors. Given the lack of curative options for patients with ATC, sorafenib warrants further study as a therapeutic agent against ATC. [Mol Cancer Ther 2007;6(6):1785–92]

Introduction

Anaplastic thyroid carcinoma (ATC), which accounts for 1.6% of all thyroid cancers, is one of the most aggressive human malignancies (1, 2). The average length of survival following diagnosis is only 4 to 6 months (2, 3), and the disease is usually well advanced by the time of diagnosis, as evidenced by the average presenting tumor size of ~8 cm. Ninety percent of patients with ATC have extraglandular spread at the time of diagnosis, and 75% of the patients will develop distant metastasis during their disease course (4, 5). Because ATC is such a lethal disease, all cases of ATC are staged by the American Joint Commission on Cancer as stage IV, regardless of tumor size, cervical lymph node status, or metastatic status (6). The treatment of ATC is frequently multimodal, and it remains controversial whether primary chemotherapy/radiotherapy results in longer survival than primary surgical intervention. Regardless, it is clear that no effective therapeutic regimen currently exists for ATC. This may be due in part to the rarity of this disease but largely reflects the inadequacy of the available treatment options and suggests an urgent need for development of novel treatment strategies.

Targeted molecular therapy and antiangiogenic therapy are rapidly becoming accepted as established anticancer treatment strategies and have shown promising results in several types of cancer (7). Sorafenib, a multikinase inhibitor produced by Bayer Pharmaceutical Corp., has both direct antitumor and antiangiogenic properties (8). These effects are mediated in part through inhibition of the BRaf kinase. In addition to the direct antitumor activity of sorafenib, it has also been shown to have antiangiogenic properties via the inhibition of vascular endothelial growth factor receptor-2 (VEGFR-2) and platelet-derived growth factor receptor-β (PDGFR-β) transmembrane receptor kinases. The effects of antiangiogenic compounds, such as AEE788 and PTK787, on thyroid cancer have been examined previously by several authors, and this approach has been suggested to be a valid anticancer strategy (9–11). The antitumor effects of sorafenib have been studied in a s.c. xenograft model of thyroid cancer. However, the effects of sorafenib have not been studied in an orthotopic model of thyroid cancer. This is an important issue as the use of an orthotopic model would
allow the evaluation of sorafenib against the endothelium of the thyroid gland.

The aim of the present study was to investigate the antitumor mechanism of sorafenib using an orthotopic model of ATC in nude mice. In this study, we found that sorafenib exerts \textit{in vitro} and \textit{in vivo} cytostatic effects on thyroid carcinoma cells. More importantly, we found that administration of sorafenib significantly inhibited the angiogenesis and growth of orthotopic ATC xenografts in nude mice and significantly improved the survival of the test animals.

Materials and Methods

Animals

Male athymic nude mice, ages 8 to 12 weeks, were purchased from the animal production area of the National Cancer Institute-Frederick Cancer Research and Development Center. The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the Assessment and Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture, the U.S. Department of Health and Human Services, and the NIH. The mice were used in accordance with the Animal Care and Use Guidelines of The University of Texas M. D. Anderson Cancer Center under a protocol approved by the Institutional Animal Care and Use Committee.

Cell Lines and Culture Conditions

ATC cell lines ARO, DRO, C643, Hth-74, and K-18 were used. Cell lines ARO, DRO, and C643 carry the \textit{BRAF}^{V600E} mutation, whereas the cell lines Hth-74 and K-18 carry wild-type \textit{BRAF}. The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, sodium pyruvate, and nonessential amino acids. Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% carbon dioxide and 95% air. The cultures were free of \textit{Mycoplasma} species. The cultures were maintained no longer than 12 weeks after recovery from frozen stocks.

Reagents

Sorafenib was generously provided by Bayer Pharmaceutical. For \textit{in vitro} administration, sorafenib was dissolved in DMSO (Sigma-Aldrich Corp.) to a concentration of 10 mmol/L and further diluted to appropriate final concentration in RPMI 1640 with 10% fetal bovine serum. DMSO in the final solution did not exceed 0.2% (v/v). For \textit{in vitro} testing, sorafenib was dissolved in Cremophor EL/ethanol (50:50; Sigma Cremophor EL, 95% ethanol) at 4× concentration. This 4× solution was prepared fresh every 4 days. Final dosing concentration was prepared by diluting the 4× solution to 1× with sterile water. The 1× solution was prepared just before it was given to the mice. Propidium iodide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were both purchased from Sigma-Aldrich.

Effects of Sorafenib on Proliferation of ATC Cell Lines

To examine the ability of sorafenib to inhibit the proliferation of ATC cell lines \textit{in vitro}, we used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based assay. Two thousand cells per well were grown in RPMI 1640 supplemented with 10% fetal bovine serum in 96-well tissue culture plates. After 24 h, the cells were treated with various concentrations of sorafenib (up to 10 μmol/L) in RPMI 1640 supplemented with 2% fetal bovine serum. Because of the concern that the DMSO in the sorafenib preparation could affect the experiments, the concentration of DMSO in all the wells was standardized and kept below 0.2% (v/v). To measure the number of metabolically active cells after a 3-day incubation period, we used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay measured by a 96-well microtiter plate reader (MR-5000; Dynatech Laboratories, Inc.) at an absorbance of 570 nm. These experiments were done at least in triplicates.

Effect of Sorafenib on the Apoptosis of ATC Cell Lines

To measure cell death, ATC cell lines were plated at a density of 2 × 10^5 per well in 38-mm² six-well plates (Costar) and maintained for 24 h before treatment with sorafenib. After 24 h, sorafenib was added in various concentrations in RPMI 1640 supplemented with 2% fetal bovine serum. The concentrations of DMSO in all the wells were adjusted to a common concentration that was always kept below 0.2% (v/v). After 48 h of treatment with sorafenib, cell death was measured using propidium iodide staining of hypodiploid DNA. The treated cells were resuspended in a Nicoletti buffer (50 mg/mL propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) for 20 min at 4°C. Cells were then analyzed by flow cytometry, and the sub-G₀-G₁ fraction was measured. These experiments were done at least in triplicates.

Western Immunoblotting

To show that sorafenib is able to inhibit the phosphorylation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase (MEK) and MAPK \textit{in vitro}, Western immunoblotting was done. DRO cells were incubated in serum-free medium for 24 h and then incubated with sorafenib for 2 h at concentrations ranging from 0.1 to 15 μmol/L before the addition of epidermal growth factor (30 ng/mL) for 15 min. The cells were then washed with PBS, and lysis buffer was added [1% Triton X-100, 20 mmol/L Tris (pH 8.0), 137 mmol/L sodium chloride, 10% glycerol (v/v), 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L aprotinin-leupeptin-trypsin inhibitor, 2 mmol/L sodium orthovanadate]. The cells were scraped and centrifuged to remove insoluble proteins. The samples were diluted in sample buffer [10% SDS, 0.5 mmol/L Tris-HCl (pH 6.8), 1 mol/L DTT, 10% (v/v) glycerol, and 1% bromphenol blue] and boiled. The proteins (50 μg) were resolved by PAGE and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) nonfat milk and 0.1% Tween 20 (v/v) in TBS and probed with the following antibodies: rabbit anti–phosphorylated MEK (pMEK) antibody (1:2,000; Cell Signaling), rabbit anti–phosphorylated MAPK (pMAPK)
antibody (1:3,000; Cell Signaling), rabbit anti-MEK antibody (1:3,000; Cell Signaling), and mouse anti-MAPK mouse antibody (1:2,000; Cell Signaling). Goat anti-rabbit IgG antibody conjugated to horseradish peroxidase and anti-mouse IgG antibody conjugated to horseradish peroxidase (Amersham Life Science, Inc.) were used (1:3,000) as the secondary antibodies. Protein bands were visualized using the Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Life Science).

**In vivo Effects of Sorafenib on Orthotopic ATC Xenografts in Nude Mice**

Orthotopic xenografts in nude mice were established as described previously (12). Briefly, DRO cells were harvested from subconfluent cultures by trypsinization and washed; 5 × 10^5 DRO cells in a volume of 5 μL were injected into the right thyroid lobe of each mouse. The tumors were allowed to develop for the next 4 days. The mice were then randomized into four groups (12 mice in each group) that received the following interventions: (a) untreated group, (b) sorafenib, 40 mg/kg, via p.o. gavage daily, or (c) sorafenib, 40 mg/kg, via p.o. gavage daily, or (d) sorafenib, 80 mg/kg, via p.o. gavage daily.

The mice were treated for 16 days and weighed twice weekly. After the treatment period, the mice were killed by CO₂ asphyxiation, and necropsy was done. At the time of necropsy, the tumors were measured in all three dimensions. The volumes of the tumors were calculated using the formula \(V = \frac{4}{3}\pi XYZ\), where \(X\), \(Y\), and \(Z\) represent the radius of the tumor in each dimension. The percentage of tumor inhibition was calculated according to the formula \(\frac{V_T}{V_C}\) × 100, where \(V_T\) and \(V_C\) represent the mean tumor volumes of the treatment group and the control group, respectively.

**Effects of Sorafenib on the Survival of Nude Mice Bearing Orthotopic ATC Xenografts**

Orthotopic ATC xenografts were established in nude mice as described above. Four days after the tumor cell injection, the mice were randomized into four groups (10 mice in each group): (a) untreated group, (b) placebo group treated with 250 μL of 1× solvent via p.o. gavage daily, (c) sorafenib, 30 mg/kg, via p.o. gavage daily, and (d) sorafenib, 60 mg/kg, via p.o. gavage daily. The mice were weighed twice weekly and killed if they lost >20% of their body weight or seemed moribund. The mice were treated for 42 days.

**Immunohistochemical Analysis of Murine Tumor Tissue Sections**

Immunohistochemical analysis was done with the following antibodies: rabbit anti-pMEK (1:100), mouse anti–proliferating cell nuclear antigen (PCNA; 1:200; DakoCytomation), rabbit anti–phosphorylated PDGFR-β (pPDGFR-β; 1:100; Santa Cruz Biotechnology), rabbit anti–phosphorylated VEGFR-2 (pVEGFR-2; tyrosine 1045; Santa Cruz Biotechnology), and rat anti-mouse CD31 (1:800; PharMingen) antibodies; goat anti-rat antibody conjugated to Alexa Fluor 594; and goat anti-rabbit antibody conjugated to Alexa Fluor 488. In addition, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was used to ascertain any intratumoral apoptosis.

For PCNA and pMEK staining, paraffin-embedded sections were first dewaxed in xylene. Excess xylene was removed by washing the slides in ethanol. After treating the tissue with pepsin for 20 min at 37°C, the slides were washed with PBS. Endogenous blocking was done with 3% hydrogen peroxide followed by protein blocking using 5% horse serum with 1% goat serum (protein-blocking solution). The primary antibodies were then added for 18 h at 4°C. The slides were then washed with PBS, blocked again with protein-blocking solution for 1 h, and incubated with horseradish peroxidase–conjugated antirabbit antibody at 1:200 dilutions for 1 h at room temperature. The slides were washed again in PBS thrice and then incubated with 3,3’-diaminobenzidine for 10 min. After the excess 3,3’-diaminobenzidine was washed off, counterstaining was done with Gill’s #3 hematoxylin.

To determine the degree of tumor angiogenesis, the tumor sections were stained for CD31. Frozen tumors were sectioned (8–10 micron thick), mounted on positively charged Superfrost slides (Fisher Scientific), air dried for 30 min, and fixed in cold acetone for 10 min. The slides were washed thrice with PBS (pH 7.5), blocked for 20 min at room temperature in protein-blocking solution, and incubated with rat anti-mouse CD31 antibody for 18 h at 4°C. The samples were then washed with PBS and blocked with protein-blocking solution for 10 min. Following the blocking step, the slides were incubated with goat anti-rat horseradish peroxidase for 1 h at room temperature. Positive staining was then visualized by incubating the slides in 3,3’-diaminobenzidine chromogen. After the excess 3,3’-diaminobenzidine was washed off, counterstaining was done with Gill’s #3 hematoxylin.

For double staining with CD31-pPDGFR-β, CD31-pVEGFR-2, and CD31-TUNEL, the frozen tumors were cut and fixed as described above. The slides were blocked for 20 min at room temperature in protein-blocking solution and incubated with rat anti-CD31 antibody for 18 h at 4°C. The slides were then washed with PBS, blocked with protein-blocking solution for 10 min, and incubated with anti-CD31 antibody conjugated to Alexa Fluor 594 for 1 h at room temperature. For CD31-pPDGFR-β and CD31-pVEGFR-2 double staining, the slides were then washed in PBS, blocked with the protein-blocking solution, and incubated with either anti-pPDGFR-β or anti-pVEGFR-2 antibody for 18 h at 4°C. After incubation, the slides were washed in PBS, blocked with the blocking solution for 10 min, and incubated with Alexa Fluor 488–conjugated antirabbit antibody for 1 h. The slides were then counterstained with 300 μg/mL Hoechst stain for 1 to 2 min at room temperature and mounted using propyl gallate.

For CD31-TUNEL double staining, TUNEL staining was done on slides already labeled with anti-CD31 antibody, as described above. TUNEL staining was done using an apoptosis detection kit (Promega) with the following modifications: tissues were fixed with 4% paraformaldehyde.
(methanol free) for 10 min at room temperature, washed twice with PBS for 5 min, and then incubated with 0.2% Triton X-100 for 15 min at room temperature. Then, the tissue sections were incubated with reaction buffer containing 44 μl equilibration buffer, 5 μl nucleotide mix, and 1 μl terminal deoxynucleotidyl transferase at 37°C for 1 h. The reaction was terminated by immersing the samples in 2x SCC for 15 min. The samples were then washed with PBS to remove unincorporated fluorescein-dUTP and mounted using propyl gallate.

Immunofluorescence microscopy was done using a Zeiss Axioplan 2 microscope (Carl Zeiss) equipped with a 100-watt HBO mercury bulb and filter sets (Chroma, Inc.) to individually capture red and blue fluorescent images. Images were captured using a CS810 Hamamatsu color chilled three-chip charge-coupled device camera (Hamamatsu Photonics K.K.) and digitized using Optimas imaging software (Media Cybernetics). The 3,3’-diaminobenzidine–stained paraffin sections were examined using a Microphot-FX microscope (Nikon) equipped with a three-chip charge-coupled device color video camera (Model DXC990; Sony Corp.).

CD31 and PCNA staining were quantified using computer-assisted image analysis with Image Pro Plus software (Media Cybernetics). The image analysis was done on four random 0.159-mm² fields (magnification, ×100) per slide from a total of six to seven slides per group. pMEK staining was quantified by counting the number of intensely stained cells on four random 0.159-mm² fields (magnification, ×100) per slide. The total number of cells was also counted in each field, and the number of intensely stained cells was expressed as a percentage of the total cell number. The photomontages were prepared using Photoshop software (Adobe Systems, Inc.).

Results

Sorafenib Inhibits the In vitro Proliferation of ATC Cell Lines

We first examined the antiproliferative effects of sorafenib on five ATC cell lines, three of which carry the BRAFV600E mutation (ARO, DRO, and C643). The IC₅₀ for inhibition of proliferation was in the range of 2 to 6 μmol/L for all the ATC cell lines examined (Fig. 1A). In particular, two ATC cell lines, ARO and DRO, showed the highest sensitivity to sorafenib, with the maximal inhibitory effect (≥80%) achieved at 3 μmol/L. Although three of the ATC cell lines studied carry the BRAFV600E mutation, we were unable to establish a definite correlation between the sensitivity of thyroid carcinoma cell lines to the antiproliferative effects of sorafenib and the presence of the BRAFV600E mutation.

Sorafenib Induces Apoptosis of ATC Cell Lines

In general, thyroid carcinoma cell lines were not very sensitive to the induction of apoptosis by sorafenib (Fig. 1B). The ATC cell line DRO was most sensitive to the proapoptotic effects of sorafenib at an IC₅₀ of 6 μmol/L, with the maximal effect of 60% apoptosis occurring at an IC₅₀ of 7.5 μmol/L. The other cell lines did not show apoptosis until treatment with 7 to 9 μmol/L sorafenib. Again, we did not observe a definite correlation between the sensitivity of the cell lines to sorafenib and the presence of BRAFV600E mutation.
Sorafenib Inhibits the Phosphorylation of MEK and MAPK in vitro in the ATC Cell Line DRO

Using Western blotting techniques, we examined the ability of sorafenib to inhibit the phosphorylation of kinases that are downstream of BRaf kinase in the signal transduction pathway (Fig. 1C). Because the cell line that we selected, DRO, also expresses VEGFR-2, we also examined the ability of sorafenib to inhibit the autophosphorylation of VEGFR-2. In this cell line, MAPK and MEK both showed constitutive phosphorylation in the absence of stimulation with epidermal growth factor. However, stimulation with epidermal growth factor caused a moderate increase in both MEK and MAPK phosphorylation. Sorafenib was able to inhibit the phosphorylation of MEK, but complete inhibition required relatively high concentrations (>10 μmol/L). The phosphorylation of MAPK was even less efficient, with residual phosphorylation of MAPK detectable even after 15 μmol/L sorafenib. However, complete inhibition of VEGFR-2 autophosphorylation occurred with 5 μmol/L sorafenib.

In vivo Antitumor Activity of Sorafenib in Orthotopic ATC Xenografts

Orthotopic ATC xenografts were established in athymic nude mice with the ATC cell line DRO. After a 16-day treatment period, the mice were sacrificed and the tumor volumes were measured. Sorafenib significantly inhibited the growth of orthotopic ATC xenografts when used at both 40 and 80 mg/kg (Fig. 2A and B). When compared with the untreated group, mice treated with 40 and 80 mg/kg sorafenib showed 63% and 93% inhibition of tumor growth, respectively. These reductions in tumor volume were statistically significant when compared with both the untreated and placebo-treated groups (P < 0.05).

Sorafenib Improves the Survival of Nude Mice Bearing Orthotopic ATC Xenografts

Nude mice bearing orthotopic ATC xenografts were treated with placebo only, 30 mg/kg sorafenib, or 60 mg/kg sorafenib. The mice in the control group and the placebo group had a 100% mortality rate by days 21 and 25, respectively (Fig. 3). The median survival durations for the control, placebo, 30 mg/kg, and 60 mg/kg groups were 17, 17, 36, and 40 days, respectively. The differences in survival between the treatment groups and the control groups were statistically significant when compared using the log-rank test (P < 0.001).

Sorafenib Inhibits the Phosphorylation of MEK and Cellular Proliferation in an Orthotopic ATC Xenograft

To assess the degree of BRaf kinase inhibition by sorafenib, immunohistochemical staining for pMEK was done in the orthotopic ATC xenografts. The difference in the percentage of pMEK-positive cells between the untreated and placebo-treated groups was not statistically significant. The tumor sections from mice treated with 40 and 80 mg/kg sorafenib showed moderate decreases in the percentage of pMEK-positive cells of ~28% and 48%, respectively (Fig. 4A). The tumor sections were also stained for PCNA to ascertain the degree of cellular proliferation. PCNA staining intensity did not differ significantly in the placebo-treated group and the untreated group. However, in tumor sections from mice treated with 40 and 80 mg/kg sorafenib, PCNA staining intensity decreased by 39% and...
Sorafenib Inhibits Angiogenesis in Orthotopic ATC Xenografts

Immunohistochemical staining for CD31 was done to determine the effect of sorafenib on tumor angiogenesis. Treatment with 40 and 80 mg/kg sorafenib resulted in significant decreases in tumor microvessel density of ~67% and 84%, respectively, compared with the control group ($P < 0.05$). Furthermore, the microvessels in control tumors were often dilated and tortuous in their histologic appearance. The microvasculature of tumors treated with sorafenib, however, was smaller in caliber, with a statistically significant decrease in the mean microvessel diameter ($P < 0.05$; Fig. 5A–C).

Double staining for CD31-TUNEL was done on the tumor specimens to assess the presence of endothelial apoptosis. The control and placebo-treated tumors did not show any endothelial apoptosis. However, treatment with both 40 and 80 mg/kg sorafenib resulted in significant induction of apoptosis by tumor endothelial cells (Fig. 5A). To determine the effect of sorafenib on the activation state of endothelial cell–expressed VEGFR-2 and PDGFR-$\beta$, double staining techniques against CD31-pVEGFR-2 and CD31-pPDGFR-$\beta$ were done. Tumors from control mice or mice treated with placebo showed strong colabeling of fluorescent red CD31 staining specific for endothelial cells, with fluorescent green staining of pVEGFR-2. Likewise, colabeling of fluorescent red for endothelial cells with fluorescent green for pPDGFR-$\beta$ staining was shown in tumors from control and placebo-treated mice. In contrast, autophosphorylation of endothelial cell–expressed VEGFR-2 and PDGFR-$\beta$ was significantly suppressed in tumors of mice treated with sorafenib (Fig. 5A).

Figure 4. Computer-assisted image analysis was done for pMEK and PCNA staining on the DRO tumors. Results of quantitative analysis for pMEK (A) and PCNA (B). Columns, mean; bars, SD.
Discussion
In the present study, we studied the \textit{in vitro} and \textit{in vivo} effects of sorafenib on ATC. First, sorafenib was shown to exert a cytostatic effect on ATC cell lines \textit{in vitro} at a concentration that is within plasma concentration shown in clinical trials (13). However, we could not show a correlation between BRAF mutational status and sensitivity to sorafenib. The administration of sorafenib to nude mice bearing orthotopic ATC xenografts resulted in the inhibition of growth in the tumor xenografts and prolonged the survival of the test animals. Despite the significant growth inhibition, tumors from mice treated with sorafenib only showed moderated decrease in the level of pMEK. The administration of sorafenib, however, did result in a significant decrease in the angiogenesis of orthotopic tumor xenografts. The antitumor effects of specific BRaf inhibition have been shown previously using RNA interference (9). However, our \textit{in vitro} and \textit{in vivo} data suggest that sorafenib may induce its antiproliferative effects via the inhibition of other targets in addition to BRaf kinase. The specificity profile of sorafenib shows that it is a multikinase inhibitor (8), and it has also been shown by Carломagno

![Figure 5](image-url)

**Figure 5.** Immunohistochemical staining was done on DRO tumors after 16 d of treatment with sorafenib. A, representative microphotographs of CD31, CD31-TUNEL, CD31-pVEGFR-2, and CD31-PDGFR-\(\beta\) staining. Original magnification, \(\times 100\). B, quantitative analysis of CD31 staining. C, quantitative analysis of mean microvessel diameter. Columns, mean; bars, SD.
et al. (14) to inhibit the RET tyrosine kinase. The oncologic importance of RET in the pathogenesis of medullary and papillary thyroid cancer has been well established. Its role in ATC, however, remains to be elucidated, and it is unclear whether inhibition of RET by sorfafenib accounts for the antitumor activity shown in this study.

The in vivo administration of sorfafenib resulted in a significant reduction in the growth of orthotopic xenografts yet resulted in only a moderate in vivo reduction in BRaf kinase activity (as assayed by pMEK staining). On the other hand, sorfafenib efficiently inhibited the activation of endothelial cell–expressed VEGFR-2 and PDGFR-β tyrosine kinases, induced significant endothelial apoptosis, and decreased tumor microvessel density. The magnitude of the antiangiogenic effects of sorfafenib suggests that its overall antitumor effects may be due to its antiangiogenic properties rather than its inhibition of the BRaf kinase. Taken together, our data suggest that sorfafenib is primarily an antiangiogenic agent when used in our orthotopic model of thyroid carcinoma.

It is well established that there is significant variability in the phenotypes of endothelial cells from various organs (15). The antitumor effects of sorfafenib in thyroid carcinoma have been shown previously by Salvatore et al. using a s.c. xenograft model (9). However, that study did not examine the effects of sorfafenib on tumor angiogenesis, perhaps owing to the possibility that the endothelia of the subcutis and the thyroid gland may differ in their susceptibility to various antiangiogenic compounds. The significant antiangiogenic response induced by sorfafenib in our orthotopic model suggests that the endothelium of the thyroid gland is highly susceptible to this compound. If sorfafenib is to be considered primarily an antiangiogenic agent when used against thyroid carcinomas, then its indication for use may not depend on the BRAF mutational status of these tumors.

In our in vivo study, an ATC cell line carrying the BRAFV600E mutation was used to establish the orthotopic xenografts in nude mice. We did not examine the effectiveness of sorfafenib against xenografts carrying wild-type BRAF, as these ATC cell lines show low tumorigenicity in nude mice. However, our in vitro observations suggest that the antiproliferative effects of sorfafenib do not correlate with BRAF mutational status. The predominantly antiangiogenic effects of sorfafenib also make it likely that this compound will be effective against thyroid carcinomas regardless of the BRAF mutational status. Last, we used relatively high doses of sorfafenib in this study to provide proof of principal that sorfafenib can be an effective antiangiogenic agent. It is likely that the magnitude of antitumor and antiangiogenic effects observed in this study may not be replicated in clinical settings. Therefore, these observations should be taken into consideration in the application of our data to any future clinical studies.

Several clinical trials are in progress with sorfafenib against various tumor types, including melanoma, renal cell carcinoma, and pancreatic carcinoma (16–20). In contrast to the multitude of clinical trials of sorfafenib against various solid tumors, interest in clinical trials pertaining to ATC remains low. Patients with ATC, however, have few therapeutic options, with most of these being palliative. The data presented in this study suggest that sorfafenib is a valid therapeutic agent in the treatment of ATC that warrants further investigation.

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Seungwon Kim, Yasemin D. Yazici, Gabriel Calzada, et al.


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