Targeted Therapy of VEGFR2 and EGFR Significantly Inhibits Growth of Anaplastic Thyroid Cancer in an Orthotopic Murine Model

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Abstract

Purpose: Anaplastic thyroid carcinoma (ATC) is one of the most lethal human cancers with a median survival of 6 months. The inhibition of epidermal growth factor receptor (EGFR) alone, or with VEGF receptor 2 (VEGFR2), represents an attractive approach for treatment of ATC. Several reports have examined agents that target these receptors. However, with the misidentification of as many as 60% of all commonly used ATC cell lines, the significance of these past findings is unclear.

Experimental Design: Cell lines authenticated by short tandem repeat profiling were selected to establish xenograft tumors in an orthotopic murine model of ATC. These mice were then treated with vandetanib to evaluate its effects on ATC tumor growth. Dynamic contrast-enhanced (DCE) MRI was utilized to measure the impact of vandetanib on tumor vasculature.

Results: Vandetanib inhibited tumor growth of the ATC cell lines Hth83 and 8505C in vivo by 69.3% (P < 0.001) and 66.6% (P < 0.05), respectively, when compared with control. Significant decreases in vascular permeability (P < 0.01) and vascular volume fraction (P < 0.05) were detected by DCE-MRI in the orthotopic xenograft tumors after 1 week of treatment with vandetanib as compared with control.

Conclusion: The inhibition of EGFR and VEGFR2 by vandetanib and its tremendous in vivo antitumor activity against ATC make it an attractive candidate for further preclinical and clinical development for the treatment of this particularly virulent cancer, which remains effectively untreatable. Vandetanib disrupts angiogenesis and DCE-MRI is an effective method to quantify changes in vascular function in vivo. Clin Cancer Res; 17(8): 2281–91. ©2011 AACR.

Introduction

There are approximately 38,000 new cases of thyroid carcinoma in the United States each year (1). Anaplastic thyroid cancer accounts for 2% of all thyroid cancers. Although thyroid cancer is an uncommon cause of mortality, the subset of patients with anaplastic thyroid carcinoma (ATC) has a grave prognosis with a mortality rate of almost 100%, and a median survival of 4 to 6 months (1, 2). ATC patients present with aggressive uncontrolled local neck disease, accompanied by lymph node and/or lung metastases (3, 4). Conventional ATC therapy employs a multimodal approach with the use of radiation therapy and conventional chemotherapeutic agents such as doxorubicin. The local disease burden often is so great that the surgical role is confined to tumor debulking and securing the airway (1, 2). Although hyperfractionated accelerated high-dose radiation therapy has improved local-regional control, the median ATC survival rate remains unchanged because of uncontrolled systemic metastasis (5–7). This suggests that lack of good systemic control and effective chemotherapy regimens are the weak links in the multimodal therapy approach.

Development of targeted molecular agents represents an exciting strategy for ATC treatment. Individual types of cancer express various molecules that drive cancer cell growth and survival. These molecules provide novel targets for therapy. Epidermal growth factor receptor (EGFR), a transmembrane cell-surface glycoprotein with intrinsic tyrosine kinase activity, is overexpressed in a majority of ATC. Activation of EGFR by its ligands, epidermal growth factor (EGF) or TGF-α, activates a cascade of secondary messenger molecules responsible for cell proliferation, increased cell survival, and angiogenesis. Pathways that are known to be activated by EGFR include the RAS-RAF-MEK-ERK pathway.
and VEGFR2 with the use of vandetanib, we would be able to significantly decrease ATC tumor growth in an orthotopic murine model. Specifically, we investigated the expression of VEGFR2 and EGFR in a panel of these validated ATC cell lines and the ability of vandetanib to inhibit phosphorylation of its target receptors. We also assessed the inhibition of proliferation of ATC cell lines in vitro. Additionally, we used immunohistochemistry to assess the effect of vandetanib on angiogenesis and apoptosis in vivo. To further delineate alterations in ATC tumor vasculature caused by vandetanib, the orthotopic xenograft tumors were assessed with dynamic contrast-enhanced (DCE) MRI.

Materials and Methods

Reagents
Vandetanib (AstraZeneca Pharmaceuticals) was diluted in dimethyl sulfoxide (DMSO; Sigma-Aldrich Corp.) for in vitro administration. For in vivo administration, vandetanib was dissolved in 1% Tween 80 (diluted in ddH2O).

Cell lines and culture conditions
ATC cell lines C643, Hth74 clone7, Hth7, Hth104, Hth83, and SW1736 were obtained from Dr. Nils-Erik Heldin (University of Uppsala, Sweden). 8505C was obtained from the laboratory of Dr. Gary L. Clayman, MD, who had purchased this cell line from the European Collection of Cell Cultures. Hth83 and Hth104 were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin, streptomycin, sodium pyruvate, l-glutamine, and nonessential amino acids. Hth74 clone7, SW1736, C643, 8505C, and Hth7 were grown in Eagle’s minimum essential medium supplemented with 10% FBS, l-glutamine, nonessential amino acids, penicillin, and streptomycin. Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% CO2 and 95% air. The cultures were free of Mycoplasma species and were maintained for no more than 10 passages after recovery from frozen stocks.

Hth83 was retrovirally infected with green fluorescent protein (GFP) and the firefly luciferase gene. For construction of the retroviral luciferase vector (Luc), a PCR product of luciferase cDNA was amplified from PGL3 vector (Promega) and cloned into pBMN-I-GFP (Dr. Garry P. Nolan, Stanford University) to generate pBMN-I-Luc-GFP. The pBMN-I-Luc-GFP vector was transfected into Phoenix cells to generate a Luc-expressing retrovirus that was subsequently used to infect Hth83 cells. Luc-transduced stable Hth83 cells were obtained by sorting GFP-positive cells for green fluorescence by FACScan (Becton Dickinson).
The luciferase cDNA expression vector used in the transfection of 8505C was constructed by obtaining luciferase cDNA from pGL3 vector by digesting with Smal and XbaI restriction enzymes and ligating to ECOR V and NHEI sites of pIRESneo3 vector (Clontech). After confirming DNA sequence, a stable cell line was made by transfecting the luciferase-pIRESNeo3 plasmid into 8505C cells using
Lipofectamine 2000 (Invitrogen) and selected using 600 µg/mL of G418 for 3 weeks.

**Cell line authentication**

DNA was harvested from the cell lines using a commercially available DNA purification kit (Qiagen). Harvested DNA was sent to the Genetic Resources Core Facility (Johns Hopkins University, Baltimore, MD) for STR profiling. Each cell line was maintained for no longer than 10 passages after genotyping.

**Immunoblot analysis**

Subconfluent cultures were grown for 24 hours in serum-free conditions and then treated with vandetanib for 2 hours. EGF (50 ng/mL) was added for 15 minutes. Cells were washed twice with ice-cold PBS and harvested in RIPA lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonylfluoride, 1 mol/L NaVO₃, 1× propidium iodide (PI), and 0.1% SDS]. Lysates were sonicated, placed on ice for 20 minutes, and centrifuged. Samples were diluted in sample buffer [10% SDS, 0.5 mmol/L Tris-HCl (pH 6.8), 0.1 mmol/L diithiothreitol, 10% (v/v) glycerol, 1% bromphenol blue] and boiled. Equal amounts of protein were resolved using 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Primary antibodies included: rabbit polyclonal anti-phospho-VEGFR2 (Tyr1054/Tyr1059; Novus Biologicals), rabbit polyclonal anti-phospho-EGFR (Tyr1068; Cell Signaling Technology), anti-phospho-Akt (Ser473, Cell Signaling Technology), or anti-phospho-mitogen-activated protein kinase (MAPK: Thr202/Tyr204, Cell Signaling Technology) in 5% bovine serum albumin (w/v) in 0.1% (v/v) Tween 20 in 1X TBS. Antibodies were visualized using either the Odyssey infrared imaging system (LI-COR Biosciences) or ECL reagent (SuperSignal West chemiluminescent system (Pierce Biotechnology). Membranes were stripped and reprobed for β-actin to verify equal protein loading.

**Measurement of cell proliferation**

ATC cells lines were plated in 96-well plates at 3,000 cells/well in 100 µL of 10% FBS media. After 24 hours of incubation, the cells were treated with vandetanib (0.59–150 µmol/L) or DMSO alone for 72 hours. MTT assay was done and absorbance was measured at 570 nm with an EL-808 96 well plate reader (Bio Tek Instruments). A separate replicate of untreated cells, representing the number of viable cells at time zero (G₀) was set aside and read at 24 hours.

**Animals and maintenance**

Male athymic nude mice, age 8 to 12 weeks, were purchased from the animal production area of the NCI-Frederick Cancer Research and Development Center (Frederick, MD). The mice were fed irradiated mouse chow and housed in laminar flow cabinets under specific pathogen-free conditions. Facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the US Department of Agriculture, the U.S. Department of Health and Human Services, and the NIH. Procedures and handling were in accordance with the Animal Care and Use Guidelines of the University of Texas MD Anderson Cancer Center (Houston, TX) under a protocol approved by the Institutional Animal Care Use Committee.

**Establishment of an orthotopic nude mouse model of ATC and treatment**

8505C-lucif and Hth83-lucif were injected (5 × 10⁵ cells/mouse or 2.5 × 10⁵ cells/mouse, respectively) orthotopically into the right thyroid gland of male athymic nude mice as previously described (19). The tumors were allowed to establish for 5 to 7 days and the mice were imaged using the IVIS 200 imaging system (Xenogen Corp.) to assess tumor size. Mice were randomized into 2 groups with equivalent mean bioluminescence values. Mice with 8505C-lucif tumors were treated with 50 mg/kg vandetanib daily by oral gavage for 4 weeks, whereas the mice with Hth83-lucif tumors were treated with 25 mg/kg daily for 3 weeks. Control mice were treated with 150 µL 1% Tween 80 in PBS. Mice were euthanized if they lost more than 20% of their body weight or became moribund. At the end of treatment, the mice were asphyxiated with CO₂, and necropsy done. At the time of necropsy, the tumors were measured in 3 dimensions and the volume was defined as V = X / (π/6), in which X, Y, and Z represent the radius of the tumor in each dimension.

**Bioluminescence imaging of orthotopic thyroid tumors**

The mice were anesthetized using 2% isofluorane (Abbott). An aqueous solution of α-luciferin (1 mg/mL; Xenogen Corp.) was injected at 150 mg/kg, 5 minutes before imaging. The mice were imaged using the IVIS 200 imaging system (Xenogen Corp.) as an overlay on IGOR software (WaveMetrics). A standardized rectangular region encompassing the head and neck of the mouse in a dorsal position was used to measure the bioluminescence. The animals were imaged on day 0 and on a weekly basis.

**DCE-MRI imaging**

Twenty mice were injected orthotopically with 1 × 10⁵ Hth83-lucif into the right thyroid gland. Tumors were grown to ensure adequate lesion size for imaging. Luciferase activity was determined and mice were randomized into 2 groups: vandetanib (25 mg/kg daily) and vehicle only. Animals were scanned weekly using the IVIS 200 Imaging System. Mice were imaged using DCE-MRI to establish baseline measurements of microvascular function (day –1). Treatment started the following day (day 0). The mice were then imaged using DCE-MRI, 1 day after therapy...
(day +1) and then again on day +7 and day +14. All data were acquired on a 4-channel Biospec 4.7T small animal MR scanner (Bruker Biospin MRI), with 26-cm imaging gradients. Mice were anesthetized using 0.5% to 2.0% isoflurane in oxygen, and a heparinized catheter was inserted into the tail vein. Four animals at a time were placed into an exchangeable multianimal positioning sled that integrated into an array of 35-mm linear volume resonators (20). A 2-plane T$_2$-weighted imaging sequence (TE = 57.7 milliseconds, TR = 2,000 milliseconds, FOV = 20 × 10 cm$^2$ over a 256 × 256 matrix) was used to confirm positioning of animals, and T$_2$-weighted coronal images (TE = 80 milliseconds, TR = 4,000 milliseconds, FOV = 6 × 3 cm$^2$ over a 256 × 192 matrix) were used to locate the tumor within each animal. An axial saturation-recovery sequence (TE = 80 milliseconds, TR = 350–4,000 milliseconds, FOV = 3 × 3 cm$^2$ over a 128 × 128 matrix) was used to measure the intrinsic T$_1$ of tumor tissue, prior to administration of contrast. Dual-tracer dynamic measurements were made using multiple repetitions of a T$_1$-weighted fast, spoiled gradient echo sequence (TE = 2.77 milliseconds, TR = 1,000 milliseconds, FOV = 3 × 3 cm$^2$ over a 96 × 96 matrix, 50-degree excitation angle; ref. 21). After 1 minute of baseline scans, 0.2 mmol/L (Gd)/kg PG-Gd-DTPA, a blood-pool contrast agent (22), was injected via the tail-vein catheter. Five minutes later, 0.2 mmol/L/kg of Gd-DTPA (Magnevist) was injected.

**Immunohistochemical analysis of CD31**

Four representative frozen tumor sections (8–10 mm) from each group were mounted on positively charged Superfrost slides (Fischer Scientific) and fixed in cold acetone. The slides were washed in PBS and incubated in endogenous peroxidase (3% H$_2$O$_2$) and protein blocking solution (5% normal horse serum, 1% normal goat serum in PBS). The samples were then incubated with rat anti-mouse CD31 antibody (BD Biosciences Pharmingen), washed in PBS, blocked with protein block, followed by secondary antibody (goat anti-rat HRP; Jackson Immunoresearch Laboratories). After washing the samples in PBS and Brij pH 7.6, the samples were incubated in Chromogen DAB (Research Genetics), washed in ddH$_2$O, counterstained with Gills #3 Hematoxylin, and mounted using Universal mount.

**Immunofluorescence double staining for CD31/terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling**

Four representative frozen tumor sections were fixed and blocked as above, excluding the endogenous peroxidase step. The slides were incubated with rat anti-mouse CD31 monoclonal antibody (BD Biosciences Pharmingen), washed with PBS, blocked with protein block and incubated with goat anti-rat Alexa 594 antibody (Invitrogen). To stain for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), a commercially available Apoptosis Detection kit (Promega) was used. Slides were washed with PBS and counterstained with 1:10,000 Hoechst stain (Invitrogen) and mounted using Vectashield Mounting Medium (Vector laboratories Inc.).

Immunofluorescence microscopy was carried out using a Leica DMLA microscope (Leica Microsystems). The microscope was equipped with a 100-W HBO mercury bulb and filter set (Chroma Inc.) that captures red and blue fluorescent images individually. Images were captured using a cooled charge-coupled device Hamamatsu 5810 camera (Hamamatsu Corp.) and Image Pro Plus 6.0 software (Media Cybernetics). Color images were captured using the same microscope equipped with a 3-chip charge-coupled device color camera (model DXC-990; Sony Corp.).

**Quantification of MVD and apoptotic endothelial cells**

Four slides from each group were selected and quantification of apoptotic endothelial cells was expressed as the average of ratio apoptotic endothelial cells to the total number of endothelial cells in four 0.04-mm$^2$ fields at a 200× magnification per slide. To quantify MVD, vessels were completely stained with anti-CD31 antibodies and counted at 10 random 0.04-mm$^2$ fields at original magnification of 200× per slide.

**Statistical analysis**

To calculate the concentration of vandetanib that caused 50% inhibition of proliferation (GI$_{50}$), we subtracted the optical density (OD) measured at 72 hours from the OD measured at G$_{0}$. GraphPad Prism 5 (GraphPad Software) software was used to plot the GI$_{50}$ by using nonlinear curve fit and variable slope, constraining the bottom to 0, using log concentrations of vandetanib. Differences in tumor volumes and immunohistochemical expression of TUNEL and CD31 from the vandetanib and control group were compared using an unpaired Student’s t-test analysis with Welch’s correction. Differences in imaging-based parameters were assessed with the Wilcoxon rank-sum test. Values of $P < 0.05$ were considered statistically significant. GraphPad Prism 5 and MATLAB (The MathWorks) were used for statistical analysis.

**Results**

**EGF stimulated the phosphorylation of EGFR and VEGFR2 and downstream signaling pathways in a panel of ATC cell lines**

After utilizing STR profiling to confirm that our cell lines were of ATC origin (Supplementary Table S1), we evaluated these cell lines for the expression and EGFr-induced phosphorylation of EGFR and VEGFR2. Specifically, a panel of 6 ATC cell lines was examined by Western blot analysis, and all were found to express both the VEGFR2 and EGFr receptors (Fig. 1A). EGFr and VEGFR2 were minimally phosphorylated after serum starvation for 24 hours. However, in response to 15 minutes of EGF stimulation (50 ng/ml), both EGFr and VEGFR2 became phosphorylated, as did the downstream signaling kinases AKT and MAPK.
Vandetanib inhibited phosphorylation of EGFR and VEGFR2 in a dose-dependent manner in vitro

To examine the ability of vandetanib to inhibit phosphorylation of EGFR and VEGFR2, we serum starved the ATC cell line 8505C, then treated it with increasing doses of vandetanib, followed by 15 minutes of EGF stimulation (50 ng/mL). We found that vandetanib inhibited EGF stimulated phosphorylation of EGFR and VEGFR2, as well as AKT and MAPK, in a dose-dependent manner (Fig. 1B).

Vandetanib inhibited proliferation of ATC cell lines in vitro

A panel of 7 ATC cell lines was incubated in increasing concentrations of vandetanib (0.59–150 μmol/L) in media supplemented with 10% FBS. Proliferation was measured using an MTT assay. After 72 hours, the proliferation of ATC cell lines was inhibited in a dose-dependent manner, with mean GI50 values ranging from 3.30 to 16.98 μmol/L (Table 1, Supplementary Fig. S1). However, when we conducted a propidium iodide (PI) assay with flow cytometry and cell-cycle analysis in cell lines Hth83 and 8505C treated with various doses of vandetanib (3–9 μmol/L), we did not see significant apoptosis in vitro (results not shown).

Inhibition of EGFR and VEGFR2 significantly reduced orthotopic ATC xenograft growth

To establish the ability of vandetanib to inhibit growth of ATC in vivo, we generated orthotopic ATC xenografts using cell lines expressing the luciferase gene. This allowed us to measure bioluminescent activity as a surrogate for tumor growth (Fig. 2). Orthotopic thyroid tumors are not palpable until they grossly compress vital structures. By measuring the bioluminescence activity within each tumor of mouse, we were able to identify the presence of tumor at an earlier stage and, more accurately, randomize the mice into groups with similar size tumors. Once treatment started, we conducted weekly bioluminescence quantification. This allowed us to follow tumor growth without having to rely on the less reliable external caliper method of measuring these highly infiltrative tumors that are deeply embedded in the thyroid gland and surrounding structures.

The mice with xenografts generated from the cell line 8505C-lucif were treated with vandetanib for a total of 4 weeks at a dose of 50 mg/kg daily. On the basis of the lower GI50 of the cell line Hth83 in vitro (3.30 vs. 7.56 μmol/L), we treated the Hth83-lucif xenograft–bearing mice at a dose of 25 mg/kg daily for 3 weeks. Bioluminescence was measured on a weekly basis (Fig. 2A). At the end of the treatment period, all mice were sacrificed and tumor volume measured. Vandetanib significantly inhibited tumor growth of ATC cell lines Hth83-lucif and 8505C-lucif, with mean tumor volume decreased by 69.32% (P < 0.0001) and 66.56% (P < 0.04), respectively, when compared with control (Fig. 2C).

Table 1. Vandetanib inhibits proliferation of ATC cell lines in vitro

<table>
<thead>
<tr>
<th>Cell line</th>
<th>GI50, mean ± SD, μmol/L</th>
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<tbody>
<tr>
<td>Hth83</td>
<td>3.30 ± 0.66</td>
</tr>
<tr>
<td>C643</td>
<td>3.65 ± 1.22</td>
</tr>
<tr>
<td>8505C</td>
<td>7.56 ± 1.13</td>
</tr>
<tr>
<td>Hth74</td>
<td>8.56 ± 1.01</td>
</tr>
<tr>
<td>SW1736</td>
<td>9.05 ± 0.55</td>
</tr>
<tr>
<td>Hth7</td>
<td>9.66 ± 0.38</td>
</tr>
<tr>
<td>Hth104</td>
<td>&gt;16.98 ± NA</td>
</tr>
</tbody>
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NOTE: GI50 values are means of at least 3 independent experiments ± SE.
MVD in ATC xenografts was significantly decreased by EGFR and VEGFR2 inhibition

To evaluate whether the growth inhibitory effect of vandetanib was associated with targeting of tumor angiogenesis through VEGFR2 inhibition, tumor sections were stained with the endothelial marker CD31 antibody and the number of tumor microvessels were counted in the tumors of vandetanib and control treated animals (Fig. 3).

The vandetanib-treated tumors had a significantly reduced MVD of 5.07 ± 0.76 MVD, when compared with tumors from control mice 19.10 ± 1.48 MVD (Table 2).

EGFR and VEGFR2 inhibition induced apoptosis of ATC tumor and endothelial cells

To further clarify the mechanism of vandetanib in vivo, we double stained tumor sections with CD31 and TUNEL.
This enabled us to quantify TUNEL staining as a measure of apoptosis alone and in relation to CD31/endothelial cells. We found that vandetanib caused significant apoptosis of tumor cells when compared with control (14.13% vs. 0.53%; \( P < 0.05 \); Table 3). In tumors treated with vandetanib, blood vessels were clearly double stained with TUNEL and CD31. We did not see this double staining in the control sections. Although there seems to be a clear trend of increased apoptotic endothelial cells in tumor sections treated with vandetanib as compared with control sections, this difference did not reach statistical significance (\( P = 0.07 \); Table 2; Fig. 3).

DCE-MRI showed significant changes in vascular volume fraction and vascular permeability in orthotopic ATC xenograft tumors treated with vandetanib

To determine the effects of vandetanib on tumor vasculature in vivo, we subjected a cohort of Hth83-lucif tumor-bearing mice that were treated with vandetanib (25 mg/kg/d) or vehicle to DCE-MRI. Serial imaging of the 2 groups of mice was done at baseline (day –1) and following treatment on day 1, day 7, and day 14. The orthotopic xenograft tumors treated with vandetanib showed decreased bioluminescence over the course of the experiment (Fig. 4A), which correlated with a significant decrease in tumor volume compared with the control group at sacrifice (60% decrease in tumor volume, \( P < 0.05 \)). Parametric maps from DCE-MRI studies showed alterations in vascular permeability and vascular volume fraction (VVF; Fig. 4B). Statistically significant decreases were noted for vascular permeability at all time points compared with baseline (\( P < 0.05 \)) in the vandetanib-treated group (Fig. 4C). VVF was decreased at all times as compared with baseline for the vandetanib-treated group: day 1 (\( P = 0.0381 \)), day 7 (\( P < 0.05 \)), and day 14 (\( P < 0.05 \)). At day 1 posttreatment, vascular permeability was not significantly altered and VVF (\( P = 0.0381 \)) were significantly decreased in the vandetanib-treated group as compared with the control group. Thus, imaging-based quantifiable biomarkers showed alteration in tumor vasculature characteristics early in the course of treatment with vandetanib.

Table 2. Quantitative immunohistochemical analysis of Hth83-lucif xenografts grown orthotopically in nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
</tr>
<tr>
<td>Apoptosis %</td>
<td>0.53 ± 0.13</td>
</tr>
<tr>
<td>MVD</td>
<td>5.07 ± 0.76</td>
</tr>
<tr>
<td>Endothelial cell apoptosis %</td>
<td>0.01</td>
</tr>
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NOTE: Mean values expressed ± SE. % Apoptosis was measured as apoptotic cells/total cells in a total of sixteen 0.04-mm\(^2\) fields at an original magnification of 200\(\times\). MVD was determined by counting the total number of complete blood vessels in forty 0.04-mm\(^2\) fields at an original magnification of 200\(\times\). % Endothelial cell apoptosis was determined as endothelial cells stained positive for CD31 and TUNEL/total endothelial cells (positive for CD31) in sixteen 0.04-mm\(^2\) fields at an original magnification of 200\(\times\). \( P \) value was determined by an unpaired Student’s \( t \) test with Welch’s connection.

Figure 3. Vandetanib inhibited MVD and increased tumor cell and tumor endothelial cell apoptosis in ATC tumor grafts. Tumors were harvested after 14 days of treatment. Representative frozen sections of Hth83-lucif tumors were stained with H&E, CD31 (brown), and double stained with CD31 (red) and TUNEL (green) (H&E and CD31 sections at 100\(\times\) original magnification, TUNEL and CD31/TUNEL at 200\(\times\) original magnification).

Discussion

Anaplastic thyroid cancer is a devastating disease and has no effective treatment options. Targeted molecular therapy has improved morbidity and mortality in a number of
EGFR is an attractive potential target for ATC, as 58% to 87% of ATC tissues overexpress EGFR when compared with normal thyroid tissue (23–25). VEGFR is crucial to the development of the microcirculation that facilitates growth of most malignancies and allows continued tumor expansion (10, 11). Combined targeting of these 2 receptors yielded promising results in recent clinical studies (26). Targeting EGFR or VEGFR in combination with conventional chemotherapeutic therapies showed favorable outcomes in a number of cancers—colon and head and neck squamous cell carcinoma (9, 27, 28). The orally available TKI vandetanib, an inhibitor of VEGFR2, EGFR, and RET, produced promising results in the treatment of metastatic hereditary medullary thyroid cancer in ongoing clinical trials (29).

We employed an orthotopic murine model of ATC to study tumor growth, the impact of therapy on ATC in its native environment, and the changes in vascular characteristics measured by DCE-MRI. This model was initially developed by Kim and colleagues (19) and was recently validated using the cell line 8505C by Nucera and colleagues (30). The orthotopic model requires direct injection of ATC cells into the thyroid of mice. These tumors closely replicate the clinical behavior of ATC in humans, including rapid growth, laryngeal/tracheal invasion and compression, and metastasis to lymph nodes and lungs (31). The orthotopic model of ATC is so analogous to clinical tumor behavior that it is challenging to detect the early stages of tumor growth. The time between initial detection of tumors by palpation and tumor-related morbidity is very short. External caliper measurements, as surrogates for measurements of tumor growth, are also notoriously inaccurate. Therefore, we chose to transduce our cell lines with the firefly luciferase gene, and use bioluminescence measurements as surrogates for tumor growth.

The use of bioluminescence is well established in models for metastasis and tumor growth in other in vivo tumor models, such as mammary and colon cancer (32, 33). Monitoring tumors by bioluminescence permitted early randomization of mice. Although a number of confounding factors, such as tumor necrosis and alterations in tumor vascularity caused by a targeted agent, may affect tumor bioluminescence over time, our bioluminescence measurements corresponded well with tumor volumes at the time of necropsy.

To our knowledge, this is the first report of a TKI that targets both VEGFR2 and EGFR, and their successful growth inhibition of ATC, in an orthotopic murine model, with cell lines validated by STR sequencing. The identification of EGFR and VEGFR as potential targets in the treatment ATC has been exploited in a number of in vitro and in vivo studies in the past 5 to 10 years (15–17). These studies have reported encouraging results, with significant inhibition of tumor growth and improved survival rates. Unfortunately, the majority of these studies used human ATC cell
lines identified in a recent paper by Scweppe and colleagues as mislabeled or duplicate (18). In fact, more than half of all commonly used thyroid cancer cell lines have been discredited.

We validated each of our cell lines with STR sequencing [STR profiles are available as Supplementary Data (Supplementary Table S1)]. The profiles were then matched to published STR profiles of known ATC cell lines (18, 34) and to the American Type Culture Collection database. We strictly adhered to the United Kingdom Coordinating Committee on Cancer Research guidelines for cell culture (35). A repository of ATC tissue specimens and matched cell lines that are maintained over time is essential to promote advancement in this area. Because ATC cell lines very rarely express the thyroid cell line markers such as thyroglobulin, thyroid transcription factor-1, or any of the cytokeratins, a matched tissue/cell line bank is crucial (36).

Our findings show that vandetanib effectively inhibits phosphorylation of its target proteins EGFR and VEGFR2 in ATC in vitro. There seems to be a strong, dose-dependent antiproliferative effect of vandetanib. However, the GI50 values are moderately high (>3.30 μmol/L). This result is consistent with previously published data by Hoffman and colleagues (37), in which the antiproliferative effects of vandetanib were evaluated in a panel of thyroid cell lines. These antiproliferative effects measured in vitro are a function of the sensitivity to EGFR inhibition by vandetanib. Therefore, while inhibition of EGFR may contribute in vivo, VEGFR2 inhibition by vandetanib may play a more significant role. The importance of VEGFR2 inhibition is further substantiated by significantly decreased MVD in tumor sections treated with vandetanib and the trend toward increased endothelial cell apoptosis.

The importance of imaging and measuring tumor angiogenesis was highlighted in recent studies linking tumor perfusion with grade and prognosis in lymphoma and head and neck cancer (38–40). DCE-MRI has not been previously utilized to assess tumor angiogenesis in an orthotopic murine model of ATC. We identified statistically significant alterations in vascular permeability (days −1, +1, +7, and +14) and VVF in vandetanib-treated animals at day +7, but not at day +1, as compared with controls. These findings underscore the importance of appropriate posttreatment DCE-MRI timing. Additionally, this alteration in imaging-based variables precedes significant differences in tumor volume measured in the treatment versus control groups (46). There are ongoing investigations to determine the predictive value of these quantifiable, imaging-based biomarkers in subsequent response to treatment. The imaging findings were consistent with our immunohistochemistry studies that revealed decreased endothelial cells in the vandetanib-treated tumors. This preclinical model provides an effective platform to assess the effects of targeted therapies through traditional measures (e.g., tumor volume) and imaging-based parameters that reflect alterations in the tumor microenvironment. Additionally, this preclinical platform enables evaluation of the effectiveness of a combination of targeted therapies with conventional modalities, such as external beam radiation therapy. This orthotopic xenograft model of ATC can be used to assess the specific sequencing of a variety of treatments to optimize potential regimens for use in clinical trials and to determine the appropriate timing for imaging studies to identify alterations in imaging-based variables that may reflect significant alterations in the tumor microvasculature. Preclinical therapeutic sequencing based on this model may provide insights that accelerate clinical care improvement. Currently, such improvement might be delayed because of the relatively low incidence of ATC, and the challenge of recruiting a sufficient number of patients to clinical trials.

In summary, we found that inhibition of EGFR and VEGFR2 in ATC by vandetanib causes significant tumor growth inhibition in vivo in an orthotopic xenograft model. This effect most likely results from the antiangiogenic effects of this agent. We found that DCE-MRI is a valuable tool in the quantifiable evaluation of ATC tumor vasculature change in vivo. We have developed a preclinical platform for assessing the sequencing and effectiveness of novel therapies for ATC. Our platform capitalizes on assessment of alterations in tumor microvasculature through noninvasive imaging studies. On the basis of these new findings, further clinical development of antiangiogenic agents such as vandetanib in the treatment of ATC is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Targeted Therapy of VEGFR2 and EGFR Significantly Inhibits Growth of Anaplastic Thyroid Cancer in an Orthotopic Murine Model

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