GROWTH INHIBITION OF ORTHOTOPIC ANAPLASTIC THYROID CARCINOMA XENOGRAFTS IN NUDE MICE BY PTK787/ZK222584 AND CPT-11

Seungwon Kim, MD,1 Yasemin D. Yazici, MD,1 Samantha E. Barber, BS,1 Samar A. Jasser, BS,1 Mahitosh Mandal, PhD,1 B. Nebiyou Bekele, PhD,2 Jeffrey N. Myers, MD, PhD1,3

1 Department of Head and Neck Surgery, Unit 441, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030–4009. E-mail: jmyers@mdanderson.org
2 Department of Biostatistics and Applied Mathematics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas
3 Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

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Abstract: Background. A preclinical evaluation of CPT-1 (Camptosar, irinotecan) and PTK787/ZK222584, a vascular endothelial growth factor receptor (VEGFR-2) tyrosine kinase inhibitor, as therapeutic agents against anaplastic thyroid carcinoma (ATC) was performed in vitro and in an orthotopic model of ATC in nude mice.

Methods. The cytotoxic and cytostatic effects of CPT-11 on ATC cell lines were evaluated. The antitumor effects of CPT-11 in combination with PTK787/ZK222584 on orthotopic ATC xenografts in nude mice were also studied.

Results. CPT-11 demonstrated significant antiproliferative effects on ATC cell lines. In vivo, PTK787/ZK222584, CPT-11, and the two agents together produced 61%, 82%, and 89% decrease in tumor growth, respectively. The differences in tumor volume between CPT-11 and CPT-11 + PTK787/ZK222584 groups were not statistically significant. PTK787/ZK222584 inhibited the phosphorylation of VEGFR-2 on tumor endothelium and decrease the tumor microvessel density.

Conclusions. The camptothecin class of chemotherapeutic agents and antiangiogenic agents such as PTK787/ZK222584 warrant further study as novel therapeutic agents against ATC.

Keywords: angiogenesis; camptothecin; irinotecan; topoisomerase; targeted molecular therapy; orthotopic model

Correspondence to: J. N. Myers

Targeted Molecular Therapy of Anaplastic Thyroid Carcinoma

Carcinomas of the thyroid gland account for approximately 1% of all new malignant diseases in the United States.1 Relatively high cure rates can be achieved in well-differentiated thyroid carcinomas such as papillary and follicular thyroid carcinoma. However, anaplastic thyroid carcinoma (ATC), one of the most aggressive human malignancies known, carries a grave prognosis.2 Although ATC accounts for only 1.6% of all thyroid cancers, the median overall survival after diagnosis is only 6 months.3,4 The disease is usually well advanced by the time of diagnosis, as evidenced by the average presenting tumor size of approximately 8 cm. Ninety percent of the pa-
tients have extraglandular spread at the time of diagnosis, and distant metastasis develops in 75% of the patients. Because ATC is such an aggressive disease, it is staged by the American Joint Committee on Cancer as stage IV regardless of tumor size, cervical lymph node status, or metastatic status.

There is no known effective cure for ATC. This may be due in part to the rarity of this disease and hence the lack of sufficient research effort. Nevertheless, the inadequacy of the current available treatment options suggests an urgent need for the development of novel treatment strategies.

Anticancer strategies that target tumor angiogenesis have shown promising results against various solid tumors in preclinical and clinical studies. In the case of ATC, several preclinical studies have shown that antiangiogenic approaches may be a valid therapeutic strategy. Antiangiogenic approaches using a monoclonal antibody against vascular endothelial growth factor (VEGF) and a small molecule inhibitor of vascular endothelial growth factor receptor (VEGFR) tyrosine kinase have been shown to decrease the growth of subcutaneous ATC xenografts in nude mice. Although these studies demonstrated the therapeutic potentials of antiangiogenic agents against ATC, the conclusions that can be drawn from these studies are limited because of the use of ectopic tumor xenograft models. It has been shown previously that the microenvironment of the subcutis is very different from that of the organs where tumors originate. Furthermore, the endothelium of various tissues demonstrates different phenotypes and may show different susceptibilities to antiangiogenic agents. For this reason, the use of orthotopic tumor models is more appropriate in the evaluation of antitumor strategies that target the endothelial compartment of the tumor. We have recently developed an orthotopic model of ATC in nude mice that accurately recapitulates the clinical and pathologic features of ATC in humans. Using this model, we have previously studied the effects of AEE788, a dual inhibitor of the epidermal growth factor receptor (EGFR) and VEGFR tyrosine kinases, on ATC and have found that this agent exerts significant antitumor effects on the growth of the orthotopic ATC xenografts in nude mice.

In this study, we evaluated the effects of an antiangiogenic agent, PTK787/ZK222584, which is a small molecule inhibitor of the VEGFR-2 tyrosine kinase, on orthotopic xenografts of ATC in nude mice. To target both the tumor cell and endothelial compartment of ATC tumors, we chose to study PTK787/ZK222584 in combination with CPT-11 (Camptosar, irinotecan). CPT-11, a member of the camptothecin class of antineoplastic agents, acts by inhibiting topoisomerase I, an enzyme that plays a critical role in DNA replication by preventing excessive supercoiling of the replicating DNA. CPT-11 is currently under development as a chemotherapeutic agent for small cell lung cancer, colorectal carcinoma, and pancreatic carcinoma. Our interest in combining an antiangiogenic agent with CPT-11 is based partly on a recent clinical trial that combined bevacizumab, a monoclonal antibody to VEGF, with a traditional chemotherapeutic regimen of CPT-11/5-fluorouracil/leucovorin for patients with metastatic colorectal carcinoma. This study showed an unprecedented improvement in survival of 5 months in the group receiving bevacizumab in combination with chemotherapeutic agents compared with those who received the chemotherapeutic agents only. Despite its tremendous promise in the treatment of adenocarcinomas such as colorectal carcinoma, particularly when combined with antiangiogenic agents, the therapeutic efficacy of CPT-11 against ATC has not yet been studied. In this study, we show that PTK787/ZK222584 in combination with CPT-11 decreased the growth, incidence of cervical lymphatic metastasis, and microvessel density (MVD) of ATC xenografts growing orthotopically in athymic nude mice.

MATERIALS AND METHODS

Reagents. PTK787/ZK222584 was generously provided by Novartis Pharma AG (Basel, Switzerland). PTK787/ZK222584 is a joint development project between Novartis Pharma AG, Basel and Schering AG, Berlin. For in vivo testing, PTK787/ZK222584 was dissolved in polyethylene glycol 300 (Acros Organics, Geel, Belgium) to a concentration of 5 mg/mL. The PTK787/ZK222584 solution was then prepared just before it was administered to the mice. CPT-11 (Pharmacia and Upjohn Co., Kalamazoo, MI) was diluted in phosphate-buffered saline (PBS) to a concentration of 5 mg/mL for intraperitoneal injection. Propidium iodide (PI) and tetrazolium (MTT) were both purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Animals. Male athymic nude mice, age 8 to 12 weeks, were purchased from the animal production area of the National Cancer Institute–Frederick Cancer Research and Development
Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for 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 National Institutes of Health. The mice were used in accordance with the Animal Care and Use Guidelines of The University of Texas M. D. Anderson Cancer Center (Houston, TX) under a protocol approved by the Institutional Animal Care and Use Committee.

**Cell Lines and Culture Conditions.** ATC cell lines ARO and DRO were used. These cell lines were obtained from Sai-Ching Yeung, MD, PhD, Department of Endocrine Neoplasia and Hormonal Disorders, M. D. Anderson Cancer Center. The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 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 Mycoplasma species. The cultures were maintained no longer than 12 weeks after recovery from frozen stocks.

**Measurement of Cell Proliferation.** To test the ability of CPT-11 to inhibit the proliferation of ARO and DRO cell lines in vitro, we used an MTT-based assay. Two thousand cells per well were grown in RPMI 1640 medium supplemented with 10% FBS in 96-well tissue culture plates. After 24 hours, the cells were treated with various concentrations of CPT-11 (0–10 μM) in RPMI 1640 medium supplemented with 2% FBS. To determine the number of metabolically active cells after a 3-day incubation period, we used an MTT assay using a 96-well microtiter plate reader (MR-5000; Dynatech Laboratories, Inc., Chantilly, VA) at an optical density of 570 nm.

**Measurement of Cell Death.** To test the ability of CPT-11 to induce the apoptosis of ATC cell lines, ARO and DRO cells were plated at a density of 2 × 10^5 cells/well in 38-mm^2 six-well plates (Costar, Cambridge, MA) and maintained for 24 hours. CPT-11 was then added in various concentrations (0–18 μM) in RPMI1640 medium supplemented with 2% FBS. After 48 hours of treatment with CPT-11, the extent of cell death was determined by PI staining of hypodiploid DNA. The treated cells were resuspended in a Nicoletti buffer (50 mg/mL PI, 0.1% sodium citrate, 0.1% Triton X-100) for 20 minutes at 4°C. The cells were then analyzed for the sub-G0/G1 fraction by flow cytometry.

**DNA Fragmentation Assay.** ARO cells (3 × 10^6 per plate) were plated in 100-mm plates. After 24 hours, the cells were treated with 7 μM CPT-11 for 12, 24, 36, and 48 hours in RPMI 1640 medium with 2% FBS for 72 hours. Both floating and attached cells were then scraped and collected in medium, washed three times with PBS, and resuspended in 1 mL of lysis buffer (20 mM Tris-HCl [pH 8], 10 mM EDTA [pH 8], and 0.5% Triton X-100). After incubation on ice for 30 minutes, the lysates were spun at 12,000 rpm in a microcentrifuge for 10 minutes. Low molecular weight DNA in the supernatant was extracted with equal volumes of phenol and chloroform for 1 hour at 4°C. Ammonium acetate (2 M) was added to the aqueous phase, and the DNA was precipitated with two volumes of ethanol at −20°C overnight. The DNA was treated with RNase A (1 mg/mL) at 37°C for 1 hour. The total DNA was then resolved on a 1.5% agarose gel and visualized with ethidium bromide staining.

**Western Immunoblotting.** To demonstrate that CPT-11 activates the apoptosis-related protein caspase 3, Western blotting against this caspase was performed. ARO cells were treated with various concentrations of CPT-11 (4, 8, 12, and 24 AM) for 72 hours in RPMI 1640 medium with 2% FBS. The cells were then washed with PBS, and lysis buffer was added (1% Triton X-100, 20 mM Tris [pH 8.0], 137 mM sodium chloride, 10% glycerol [v/v], 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 mM aprotinin-leupeptin-trypsin inhibitor, 2 mM sodium orthovanadate). The cells were scraped and centrifuged to remove insoluble proteins. The samples were diluted in sample buffer (10% sodium dodecyl sulfate, 0.5 mM Tris-HCl [pH 6.8], 1M dithiothreitol (DTT), 10% [v/v] glycerol, and 1% bromophenol blue) and boiled. The proteins (100 μg) were resolved by polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) nonfat milk in 0.1% Tween 20 (v/v) in Tris-buffered saline and probed with rabbit anti-caspase 3 antibody (diluted 1:3000) (Cell Signaling, Beverly, MA) in 1% nonfat milk. The membranes were then incubated with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (IgG) (Amersham, Inc.,
Arlington Heights, IL) at 1:3000 as the secondary antibody. Protein bands were visualized by using an electrochemoluminescent (ECL) plus Western blotting detection system (Amersham).

**Effects of PTK787/ZK222584 and CPT-11 on the Growth of Orthotopic ATC Xenografts in Nude Mice.** We used an orthotopic model of ATC in nude mice for the in vivo phase of the study. It is well established that human VEGF binds and activates murine VEGFR, which then results in angiogenic responses. Furthermore, we have previously verified that human ATC cell line ARO produces and secretes VEGF (unpublished data). Therefore, we believed that the use of human xenograft tumors in a murine model for studies involving antiangiogenic compounds was valid study design. Orthotopic xenografts in nude mice were established as described previously. ARO cells were harvested from subconfluent cultures by trypsinization and washed; $5 \times 10^5$ ARO cells suspended in a volume of 5 μL were injected into the right thyroid lobes of each mouse using a 30-gauge needle. The tumors were allowed to develop during the following 4 days. The mice were then randomly assigned into four groups (10 mice per each group), and the drugs were administered as follows: (1) PTK787/ZK222584 by oral gavage at 50 mg/kg, once/day; (2) CPT-11 by intraperitoneal injection at 50 mg/kg, once/week; (3) both PTK787/ZK222584 by oral gavage at 50 mg/kg, once/day, and CPT-11 by intraperitoneal injection, 50 mg/kg, once/week; and (4) 250 μL polyethylene glycol 300 administered by oral gavage once/day and PBS administered intraperitoneally once/week as placebo.

The mice were treated for 4 weeks and weighed twice per week. Our animal protocol required that the animals be killed if they lost $>20\%$ of body weight or if they became moribund. However, all the animals maintained their weights, and none met the criteria for sacrifice before the end of the treatment period. At the end of the 4-week treatment period, the mice were asphyxiated by carbon dioxide, and necropsy was performed. The cervical lymph nodes and the lungs were removed during the necropsy, sectioned and stained with hematoxylin–eosin (H&E), and examined for the presence of metastasis. At the time of the necropsy, the tumors were measured in three dimensions. The volumes of the tumors were defined as $V = \frac{4}{3}\pi XYZ$, where ‘X,’ ‘Y,’ and ‘Z’ represent the radius of the tumors in each dimension. Of the 10 mice in each group, one from the control group and two each from the PTK787/ZK222584 and combination groups died during the treatment period because of gavage-related trauma. These mice were excluded from the calculation of average tumor volumes for each group at the end of the study. PTK787/ZK222584 and CPT-11 were administered 2 hours before the mice were killed at the end of the 4-week treatment period.

For immunohistochemical and routine H&E staining, one part of the tumor was fixed in formalin and embedded in paraffin. Another part was embedded in ornithine carbamoyltransferase (OCT) compound (Miles Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at $-80\degree$C.

**Effects of PTK787/ZK222584 and CPT-11 on the Survival of Nude Mice Bearing Orthotopic ATC Xenografts.** Orthotopic ATC xenografts were established in nude mice as described previously. However, for the survival study, the right thyroid lobe of each mouse was injected with $1 \times 10^6$ ARO cells suspended in an injection volume of 7.5 μL. Four days after injection of the tumor cells, the mice were randomly assigned into four groups: control, PTK787/ZK222584, CPT-11, and combination treatment. Each group of mice was treated with PTK787/ZK222584, CPT-11, or both agents, as described in the previous section. The mice were weighed twice per week and killed if they showed weight loss of $>20\%$ or appeared moribund. The mice were treated for 35 days.

**Immunohistochemistry on Murine Tumor Tissue Sections.** Immunohistochemistry was performed with rabbit anti-phosphorylated VEGFR-2 (pVEGFR-2) (tyrosine 1045) (Santa Cruz Biotechnology, Santa Cruz, CA) and rat anti-mouse CD31 (PharMingen, San Diego, CA) antibodies.

To quantify the MVD of tumors from the control and treatment groups, staining for intratumoral microvasculature was performed using rat anti-mouse CD31 antibody. First, frozen tumors were sectioned (8–10 μm thick), mounted on positively charged Superfrost slides (Fisher Scientific, Houston, TX), air dried for 30 minutes, and fixed in cold acetone for 10 minutes. The slides were then washed three times with PBS (pH 7.5), blocked for 20 minutes at room temperature in PBS supplemented with 1% normal goat serum and 5% normal horse serum (protein-blocking solution), and incubated with anti-CD31 antibody (1:800 dilution) for 18 hours at 4°C. The samples were then washed three times for 3 minutes and blocked...
with protein-blocking solution for 10 minutes. Horseradish peroxidase (HRP)–conjugated anti-rat antibody was then added (1:200 dilution) for 1 hour at room temperature. The slides were washed again in PBS three times and then incubated with diaminobenzidine tetrahydrochloride (DAB) for 10 minutes. After the excess DAB was washed off, counterstaining was performed with Gill’s #3 hematoxylin.

To ascertain the degree of activation of endothelium-expressed VEGFR-2, double staining for pVEGFR-2 and CD31 was performed. First, frozen tumors were sectioned (8–10 μm thick), mounted on positively charged Superfrost slides (Fisher Scientific), air dried for 30 minutes, and fixed in cold acetone for 10 minutes. The slides were then washed three times with PBS (pH 7.5), blocked for 20 minutes at room temperature in PBS supplemented with 1% normal goat serum and 5% normal horse serum (protein-blocking solution), and incubated with mouse anti-CD31 antibody (1:800 dilution) for 1 hour at room temperature in the dark. The samples were washed three times for 3 minutes and blocked with protein-blocking solution for 10 minutes. Alexa 594-conjugated goat anti-mouse IgG (1:400 dilution) (Molecular Probes, Eugene, OR) was added for 1 hour at room temperature in PBS. The slides were then washed three times, 3 minutes each time, blocked with protein-blocking solution for 20 minutes, and incubated with rabbit anti-pVEGFR-2 antibody for 18 hours at 4°C. After the overnight incubation, the slides were washed three times with PBS, 3 minutes each time, and blocked with protein-blocking solution for 10 minutes. Samples were then incubated with Alexa 488-conjugated goat anti-rabbit IgG at 1:400 (Molecular Probes, Eugene, OR) for 1 hour at room temperature in the dark. The slides were again washed three times for 5 minutes and then counterstained with 300 μg/mL of Hoechst stain for 1 to 2 minutes at room temperature. The slides were again washed and then mounted using propyl gallate.

Immunofluorescence microscopy was performed using a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) equipped with a 100-watt HBO mercury bulb and filter sets (Chroma Inc., Brattleboro, VT) to individually capture red and blue fluorescent images. The images were captured by a color chilled three-chip charge-coupled device Hamamatsu C5810 camera (Hamamatsu Photonics K.K., Bridgewater, NJ) and digitized using Optimas imaging software (Media Cybernetics, Silver Spring, MD). To image the DAB-stained sections, the stained sections were examined in a Microphot-FX microscope (Nikon, Melville, NY) equipped with a three-chip charge-coupled device color video camera (Model DXC990; Sony Corp., Tokyo, Japan).

To quantify MVD, the labeled endothelial cells were counted from five random 0.159-mm² fields (100× magnification) per slide from total of eight slides per study group. The photomontages were prepared using Photoshop software (Adobe Systems Inc., San Jose, CA).

**Statistical Analysis.** The average tumor volumes and the MVD of the control and treatment groups were compared using the independent sample t test. The incidences of cervical and pulmonary metastases were compared using the chi-square test. The survival data were analyzed by Kaplan–Meier methods, and the survival periods were compared by the log-rank test.

**RESULTS**

**CPT-11 Inhibits Proliferation of ATC Cell Line ARO In Vitro.** ATC cell lines ARO and DRO were incubated with increasing concentrations (0–10 μM) of CPT-11 in RPMI 1640 medium complemented with 2% serum. After 72 hours, the MTT assay showed that the proliferation of both ARO and DRO cells was inhibited by CPT-11 in a dose-dependent manner (Figure 1A). The concentration required to cause 50% cell growth inhibition (IC₅₀) was determined to be approximately 2 μM for both ARO and DRO. The proliferation of ARO and DRO cell lines were decreased by approximately 30% and 20%, respectively, at the IC₅₀. The maximal inhibition of proliferation of 60% and 40% was achieved at 8 μM of CPT-11. We also investigated the in vitro effects of PTK787/ZK222584 on ATC cell lines and found that PTK787/ZK222584 did not affect the proliferation of ATC cell lines (data not shown).

**CPT-11 Induces Apoptosis of ATC Cell Lines In Vitro.** To examine the proapoptotic effects of CPT-11, the ARO and DRO cells were treated with various concentrations of CPT-11 for 48 hours. PI staining showed that CPT-11 induced apoptosis in ARO cells in a dose-dependent manner (Figure 1B). The ARO cell line was more sensitive than DRO to the induction of apoptosis by CPT-11. The IC₅₀ for induction of apoptosis was found to be 7 μM for ARO and 11 μM for DRO. The magnitude of apoptosis at the IC₅₀ was approximately 35% and...
10\% for ARO and DRO, respectively. The maximal apoptosis of 70\% and 20\% for the ARO and DRO cell lines, respectively, required $>14 \mu M$ of CPT-11.

To confirm the proapoptotic effects of CPT-11 on ATC cell lines, a DNA fragmentation assay was performed on the ARO cell line. When incubated with 7 $\mu M$ of CPT-11 ($IC_{50}$ for induction of apoptosis from the PI assay), DNA fragmentation was detectable by 36 hours (Figure 2A). We also performed Western blotting for caspase-3, a marker of apoptosis. Induction of apoptosis results in cleavage of inactive caspase-3 into an active form. Incubation with 8 $\mu M$ and higher concentrations of CPT-11 for 72 hours activated caspase-3, as evidenced by the detection of cleaved caspase-3 on Western blot analysis (Figure 2B). PTK787/ZK222584, on the other hand, did not induce the apoptosis of ATC cell line when examined by PI assay (data not shown).

**PTK787/ZK222584 and CPT-11 Inhibits Orthotopic ATC Xenograft Growth In Vivo.** PTK787/ZK222584 alone and CPT-11 alone inhibited the growth of orthotopic ATC xenografts generated with the ARO cell line in nude mice. However, the highest growth inhibition was achieved by the co-administration of PTK787/ZK222584 and CPT-11 (Table 1, Figure 3). At the end of the 4-week treatment period, the mice treated with PTK787/ZK222584, CPT-11, and PTK787/ZK222584 plus CPT-11 showed 61\%, 82\%, and 89\% decreases, respectively, in mean tumor volume compared with the
control group. CPT-11 and the PTK787/ZK222584 + CPT-11 combination produced statistically significant ($p < .05$) reductions in mean tumor volume. The reduction in mean tumor volume by PTK787/ZK222584 alone, however, was not statistically significant ($p = .092$), most likely because of the relatively small number of mice in each group and the wide variation in tumor sizes within each group. The difference in mean tumor volume between the PTK787/ZK222584 group and the combination treatment group was statistically significant ($p = .029$), but the difference between

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Mean tumor volume ± SEM, mm$^3$</th>
<th>Range, mm$^3$</th>
<th>Tumor inhibition, %</th>
<th>$p$ value$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>285.71 ± 83.91</td>
<td>113.04–918.45</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PTK787/ZK222584</td>
<td>120.43 ± 33.74</td>
<td>31.40–329.70</td>
<td>61</td>
<td>.092</td>
</tr>
<tr>
<td>CPT-11</td>
<td>50.08 ± 8.81</td>
<td>18.84–109.93</td>
<td>82</td>
<td>.012</td>
</tr>
<tr>
<td>PTK787/ZK222584</td>
<td>31.03 ± 4.03</td>
<td>13.08–47.10</td>
<td>89</td>
<td>.007</td>
</tr>
<tr>
<td>+CPT-11</td>
<td></td>
<td></td>
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Abbreviation: ATC, anaplastic thyroid carcinoma.

$^*$Independent sample t test. The mean tumor volumes of the treatment groups were compared with the control group.

FIGURE 3. The effects of irinotecan (CPT-11) and PTK787/ZK222584 on the growth of orthotopic anaplastic thyroid carcinoma (ATC) xenografts in nude mice. The mice were treated with CPT-11, PTK787/ZK222584, or both CPT-11 and PTK787/ZK222584, as outlined in the “Materials and Methods” section, for 4 weeks. Photographs of representative tumors from (A) control group, (B) CPT-11 group, (C) PTK787/ZK222584 group, and (D) combination treatment group at the end of the treatment period. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
the CPT-11 group and the combination group did not achieve statistical significance ($p = .094$).

Treatment with PTK787/ZK222584 and CPT-11 also statistically significantly decreased the incidence of cervical lymph node metastasis ($p < .05$). The incidences of cervical lymph node metastasis were 77%, 37%, 20%, and 13% in the control, PTK787/ZK222584, CPT-11, and combination treatment groups, respectively. Pulmonary metastases were found in two of nine mice in the control group and in none of the mice in the three treatment groups. The differences in the rates of pulmonary metastasis were not statistically significant ($p > .05$).

Of the 10 mice in each group, one from the control group and two each from the PTK787/ZK222584 and combination treatment groups died during the treatment period because of gavage-related trauma. However, the rest of the animals tolerated both PTK787/ZK222584 and CPT-11 without significant weight loss (data not shown).

To determine the effect of PTK787/ZK222584 on the phosphorylation of VEGFR-2 expressed by the tumor endothelium, the CD31/pVEGFR-2 double labeling technique was used (Figure 4). Tumors from control mice or mice treated with CPT-11 showed co-labeling (yellow) of fluorescent red staining specific for endothelial cells with fluorescent green staining specific for pVEGFR-2. In contrast, the phosphorylation of the VEGFR-2 expressed by the tumor endothelium was significantly suppressed in tumors of mice treated with PTK787/ZK222584, either alone or in combination with CPT-11. We have found previously that the ATC cell line ARO also expressed VEGFR-2 (data not shown). In accordance with this previous finding, tumor cells from control tumors also stained positively for pVEGFR-2, and this phosphorylation was also inhibited in tumor cells

**PTK787/ZK222584 Inhibits Tumor Associated Angiogenesis and Endothelial-Cell Expressed VEGFR-2 Phosphorylation.** To determine the effect of PTK787/ZK222584 on tumor angiogenesis, MVD was quantified by staining tumor sections for CD31 (Figure 4, Table 2). Treatment with CPT-11 did not affect the tumor MVD significantly compared with the control group. However, treatment with PTK787/ZK222584, alone or in combination with CPT-11, resulted in a statistically significant decrease in tumor MVD ($p < .01$).

**Table 2. Quantitative analysis of CD31 immunohistochemical staining.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Microvessel density, mean ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.8 ± 1.6</td>
</tr>
<tr>
<td>PTK787/ZK222584</td>
<td>10.4 ± 1.5†</td>
</tr>
<tr>
<td>CPT-11</td>
<td>14.6 ± 1.6‡</td>
</tr>
<tr>
<td>PTK787/ZK222584 + CPT-11</td>
<td>11.0 ± 1.8§§</td>
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*Microvessel density was quantified by counting cells positively stained for CD31 from five random 0.159-mm² fields (100× magnification) per slide from total of eight slides per treatment group. The numerical values listed represent the mean number of vessels per unit area ± standard deviations. $p = .002$ compared with the control group (independent sample t test). $t_p = .055$ compared with the control group (independent sample t test). $t_p = .005$ compared with the control group (independent sample t test).
from mice treated with PTK787/ZK222584, either alone or in combination with CPT-11 (Figure 4).

**PTK787/ZK222584 and CPT-11 Improves the Survival of Nude Mice Bearing Orthotopic ATC Xenografts.** Treatment of nude mice bearing orthotopic ATC xenografts with PTK787/ZK222584, CPT-11, or both agents together resulted in statistically significant improvement in the survival of these animals (\(p < .05\)) (Figure 5). All of the control mice succumb to the thyroid tumors because of obstruction of the upper aerodigestive tract by day 21. The mean survival periods were 21, 27, 32, and 34 days for the control, PTK787/ZK222584, CPT-11, and the combination treatment groups, respectively.

**DISCUSSION**

Antiangiogenesis is a therapeutic strategy that has shown promising results against various malignancies in preclinical and clinical trials. This strategy has been previously explored in ATC using murine ectopic xenograft models. However, Teicher\(^2^{1}\) proposed that antiangiogenic therapy should ideally be combined with cytotoxic therapy (ie, chemotherapy or radiotherapy), because such a combination of antiangiogenic and cytotoxic agents will provide comprehensive targeting of both the cancer cell and endothelial compartment of the tumor. In this study, we investigated the effects of combined therapy with PTK787/ ZK222584, an inhibitor of the VEGFR-2 tyrosine kinase, and CPT-11 on the growth and progression of orthotopically established ATC xenografts in nude mice. Neither of these agents has been studied previously as therapeutic agents against ATC.

Although CPT-11 has not yet been evaluated for treatment of thyroid carcinoma, its role in the treatment of colorectal, ovarian, cervical, small cell lung, and non-small cell lung carcinoma has been evaluated in several studies.\(^2^{2},^{2}^{3}\) Two phase III trials showed that combining CPT-11 with 5-fluorouracil in the treatment of colorectal carcinoma had survival benefit in patients with metastatic colorectal carcinoma over treatment with either agent alone.\(^2^{2},^{2}^{3}\) Given the relentless drive for proliferation that is so characteristic of ATC, we hypothesized that CPT-11 may be effective in vitro and in vivo against this disease. In this study, we showed that CPT-11 is able to inhibit the proliferation and induce the apoptosis of ATC cell lines in vitro. The in vitro IC\(_{50}\) for antiproliferative and
proapoptotic effects of CPT-11 was within the plasma concentration achievable in human patients (3–5 μM) when given intravenously at the standard dose of 350 mg/mL.24,25 Furthermore, CPT-11 decreased tumor growth by approximately 80% and significantly inhibited the rate of lymph node metastasis when administered to nude mice bearing ATC orthotopic xenografts in nude mice as a single-agent therapy.

The antitumor efficacy of PTK787/ZK222584 against poorly differentiated thyroid tumors was demonstrated previously by Schoenberger et al,9 who showed that this agent inhibited approximately 40% of subcutaneous xenograft growth in nude mice. However, it is well established that different phenotypes are exhibited by endothelium from different tissues.13,14 Therefore, the sensitivity of the endothelial cells of the subcutis to the antiangiogenic activity of PTK787/ZK222584 may not correlate with the sensitivity of thyroid endothelium to this compound. To address this issue, we used an orthotopic model ATC in our study. In this model, PTK787/ZK222584 produced approximately 60% inhibition of tumor growth. Furthermore, we have demonstrated using staining that PTK787/ZK222584 effectively inhibits the activation of endothelial-expressed VEGFR-2 with a corresponding decrease in MVD. These data suggest that the microvasculature of thyroid glands is sensitive to the effects PTK787 and that antiangiogenic approaches may be effective therapeutic strategies for thyroid carcinomas.

The expectation of a linear correlation between the antiangiogenic effects of PTK787/ZK222584 (as reflected by the magnitude of the decrease in MVD) and the degree of tumor inhibition is somewhat simplistic. However, if we assume that the inhibition of tumor growth by PTK787/ZK222584 is purely due to its antiangiogenic property, then the magnitude of tumor inhibition achieved by PTK787/ZK222584 (approximately 60% when used as a single agent) is higher than expected for a decrease in MVD of approximately 20%. Therefore, antitumor mechanisms of PTK787/ZK222584 other than antiangiogenesis need to be considered. We have found that many ATC cell lines (including ARO, which was used to generate the orthotopic xenografts) express VEGFR-2 (unpublished data). Although the functional significance of tumor-expressed VEGFR has not yet been established, it has been proposed that these receptors may be involved in tumor establishment or progression by means of an autocrine mechanism.26,27 Although PTK787/ZK222584 did not show antiproliferative or proapoptotic effects on ATC cell lines in vitro (unpublished data), the inhibition of tumor-expressed VEGFR-2 in an in vivo setting may result in cytostatic or cytotoxic effects.

In several clinical trials, the co-administration of an antiangiogenic agent with a cytotoxic agent has often shown synergistic effects.20 However, considering the fact that the end effect of antiangiogenic agents is decreased tumor perfusion (and decreased exposure of the tumor to the drug), the synergism between antiangiogenic and chemotherapeutic agents is somewhat counterintuitive. The ability of VEGFR-inhibiting agents to normalize pathologic tumor vasculature has received much attention recently as a possible explanation for this synergism.28 The nascent tumor microvasculature is usually leaky and tortuous and results in an abnormal tumor microenvironment characterized by interstitial hypertension and hypoxia. The interstitial hypertension interferes with the delivery of chemotherapeutic agents to tumor cells, and the hypoxia fosters tumor cell resistance to chemotherapeutic agents. It has been shown previously that agents that inhibit the VEGFR pathway will normalize the leaky and saccular tumor microvascular vasculature and reverse the interstitial hypertension, hypoxia, and acidosis. As a result, the delivery and penetration of chemotherapeutic agents are improved, and tumor-cell resistance to chemotherapeutic agents is decreased.28 Yano et al29 showed that PTK787/ZK222584 decreased the pleural effusion in an orthotopic nude mice model of lung adenocarcinoma by normalizing and decreasing the permeability of the pathologic microvasculature. Further study is needed to determine whether the antitumor activity of PTK787/ZK222584 against ATC involves normalization of tumor vasculature resulting in synergy between this agent and chemotherapeutic agents such as CPT-11.

CONCLUSION

ATC is a highly aggressive disease with a poor prognosis that warrants the development of novel treatment strategies. This study has demonstrated the therapeutic potentials of camptothecin class of antineoplastic agents and antiangiogenic approaches in the treatment of ATC. Considering the fact that curative options seldom exist for patients with ATC, the camptothecins and antiangiogenic agents such as PTK787/ZK222584
REFERENCES