Published in final edited form as:

Head Neck. 2011 March ; 33(3): 349–358. doi:10.1002/hed.21455.

Targeted molecular therapy of head and neck squamous cell carcinoma with the tyrosine kinase inhibitor vandetanib in a mouse model

Daisuke Sano, MD, PhD¹, David R. Fooshee¹, Mei Zhao, MD¹, Genevieve A. Andrews, MD¹, Mitchell J. Frederick, PhD, BS¹, Chad Galer, MD¹, Zvonimir L. Milas, MD¹, Phuong Khanh H. Morrow, MD³, and Jeffrey N. Myers, MD, PhD^{1,2}

¹ Department of Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

² Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

³ Department of Breast Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Abstract

Background—We investigated the effects of vandetanib, an inhibitor of vascular endothelial growth factor receptor 2 (VEGFR-2) and epidermal growth factor receptor (EGFR), alone and in combination with paclitaxel in an orthotopic mouse model of human head and neck squamous cell carcinoma (HNSCC).

Methods—The *in vitro* effects of vandetanib (ZACTIMA™) were assessed in two HNSCC cell lines on cell growth, apoptosis, and receptor and downstream signaling molecule expression and phosphorylation levels. We assessed *in vivo* effects of vandetanib and/or paclitaxel by measuring tumor cell apoptosis, endothelial cell apoptosis, microvessel density, tumor size, and animal survival.

Results—*In vitro*, vandetanib inhibited the phosphorylation of EGFR and its downstream targets in HNSCC cells and inhibited proliferation and induced apoptosis of HNSCC cells and extended survival and inhibited tumor growth in nude mice orthotopically injected with human HNSCC.

Conclusion—Vandetanib has the potential to be a novel molecular targeted therapy for HNSCC.

Keywords

vandetanib; targeted molecular therapy; vascular endothelial growth factor receptor 2 (VEGFR-2); epidermal growth factor receptor (EGFR); head and neck squamous cell carcinoma

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCCs) represent approximately 2.4% of all cancers in the United States, where they are projected to account for 35,720 new diagnoses and 7,600 deaths in 2009 (1). Advanced stage at diagnosis and aggressive tumor biology contribute to the observed poor prognosis in newly diagnosed patients, whose 5-year

survival rates range from 10% to 40% (2). Thus, new treatment modalities are clearly needed to more effectively treat these malignancies.

Targeted molecular therapy is a promising new development in treating human cancers (3). Unlike standard cytotoxic therapies, which generally lack specificity for tumor cells, the targeted molecular approach exploits known molecular changes in neoplastic cells. Potential biological targets for HNSCC are the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor receptor 2 (VEGFR-2). EGFR, a member of the receptor tyrosine kinase superfamily of transmembrane receptor proteins, is bound and activated by its ligands, which include epidermal growth factor (EGF) and transforming growth factor- α . The EGFR tyrosine kinase domain phosphorylates several second messenger molecules, providing signals that enhance cell survival and increase cell proliferation (4). Targeted inhibition of the overexpressed EGFR in neoplastic cells can therefore hinder cell proliferation and survival, making this an attractive target for molecular therapy. VEGFR mediates normal vascular development and physiological angiogenesis. However, the VEGF and VEGFR overexpression common in HNSCC facilitates tumor angiogenesis, allowing the neoplastic tumor to grow (5). EGFR overexpression has been reported in 34% to 80% of case of HNSCCs and correlates with shortened disease-free survival in patients with HNSCC. Increased VEGF and VEGFR expression has also been observed in aggressive subsets of these HNSCCs (6–8).

To evaluate the potential efficacy of targeting both EGFR and VEGFR signaling in HNSCC treatment, we tested vandetanib (AstraZeneca), an orally bioavailable small-molecule tyrosine kinase inhibitor that selectively targets both EGFR and VEGFR-2 tyrosine kinases (9). We investigated the ability of vandetanib to inhibit EGFR and VEGFR-2 signaling and initiate an antiproliferative effect *in vitro*. We then examined its antitumorigenic and antiangiogenic capabilities *in vivo*, alone and in combination with the cytotoxic agent paclitaxel (Bristol-Myers). We hypothesized that vandetanib would inhibit cell proliferation *in vitro*, inhibit tumor growth and angiogenesis *in vivo*, and interact with paclitaxel *in vivo* to reduce tumor volume to a greater extent than vandetanib alone (10).

MATERIALS AND METHODS

Animals and Maintenance

Eight-to-12-week-old male athymic nude mice were purchased from the National Cancer Institute (Bethesda, MD). The mice were kept in a specific pathogen-free facility and were fed irradiated mouse chow and autoclaved, reverse osmosis-treated water. The facility was approved by the American Association for the Accreditation of Laboratory Animal Care and met all current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the National Institutes of Health. Animal procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center.

Cell Lines

Two human HNSCC cell lines were used in the study. The FaDu line was purchased from the American Type Culture Collection (Manassas, VA). This cell line was established in 1968 from a punch biopsy of a hypopharyngeal carcinoma. The SCC61 line was obtained from Dr. Alissa Weaver of Vanderbilt University (Nashville, TN). This cell line was isolated from tongue squamous cell carcinoma tumors (11). FaDu cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium pyruvate, nonessential amino acids, and a twofold vitamin solution (Life Technologies, Inc., Grand Island, NY). SCC61 cells were maintained in DMEM

supplemented with 20% FBS and 0.4 µg/mL hydrocortisone. Adherent monolayer cultures were maintained on plastic plates and incubated at 37°C in 5% carbon dioxide and 95% air. The cultures were free of *Mycoplasma* species and were maintained for no longer than 12 weeks after recovery from frozen stocks.

Reagents

Vandetanib (Zactima, ZD6474) was provided by AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, UK). For *in vivo* testing, vandetanib was dissolved in phosphate-buffered saline (PBS) containing 1% Tween 80. For *in vitro* testing, stock solutions of vandetanib were prepared in dimethylsulfoxide (Sigma-Aldrich Corp., St. Louis, MO) and diluted with culture medium. Paclitaxel (Taxol/Bristol-Myers Squibb, Princeton, NJ) was diluted in PBS to a 1 mg/mL final concentration. Propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were both purchased from Sigma-Aldrich Corp. (St. Louis, MO). Stock solutions were prepared by dissolving either 0.5 mg of PI or 2 mg of MTT in 1 mL of PBS. Each solution was then filtered to remove particles, protected from light, stored at 4°C, and used within 1 month.

The primary antibodies for immunohistochemical analysis were purchased as follows: rat monoclonal anti-mouse CD31 (platelet-endothelial cell adhesion molecule 1; PECAM) (BD Pharmingen, San Diego, CA). The secondary antibodies were used as follows: peroxidase-conjugated goat anti-rat immunoglobulin G1 (Jackson Research Laboratories, West Grove, PA); and Alexa Fluor 594-conjugated goat anti-rat immunoglobulin G.

Cell Proliferation Assay

The anti-proliferative ability of vandetanib against HNSCC cells *in vitro* was determined using an MTT assay as previously described (12). Briefly, FaDu and SCC61 were plated in 96-well plates at 5,000 cells per well in medium with 10% FBS and 20% FBS, respectively. After a 24-hour attachment period, the cells were incubated for 72 hours in various concentrations of vandetanib (0.3–15 µM) or with dimethylsulfoxide alone as a control. Cells were then incubated for 3 hours in medium containing 2% FBS and 0.25 mg/mL MTT, after which the cells were lysed in 100 µL dimethylsulfoxide to release the formazan. The conversion of MTT to formazan was quantified with an EL-808 96-well plate reader (BioTek Instruments, Winooski, VT) set at an absorbance of 570 nm. The concentration of vandetanib giving 50% growth inhibition (GI50) for each cell line was calculated using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA). The experiment was repeated at least twice. The vandetanib GI50 was the average of the values from each MTT assay.

Flow Cytometry for Apoptosis

FaDu and SCC61 cells (2×10^5 per well) were plated in 6-well plates (Costar, Cambridge, MA) in 2 mL medium containing 2% FBS, incubated for 24 hours, and then treated with various concentrations (2–5 µM) of vandetanib. Afterward both adherent and detached cells were harvested, washed with PBS, and resuspended in Nicoletti buffer (50 µg/mL PI, 0.1% sodium citrate, 0.1% Triton X-100, and 1 mg/mL RNase in PBS). Using the sub-G₀/G₁ fraction, we calculated the percentage of apoptotic cells by gating the hypodiploid region on the DNA content histogram with the Lysis program (Becton Dickinson, Franklin Lakes, NJ) (13).

Western Blotting of EGFR Tyrosine Kinase Inhibition by Vandetanib

FaDu and SCC61 cells were plated in 6-well plates at 1.5×10^5 cells per well and incubated in medium with 10% FBS and 20% FBS, respectively, overnight. The next day, cells were washed with PBS and incubated in serum-free medium for 24 hours. Cells were then treated

for 90 minutes with 0–10 μM vandetanib in dimethylsulfoxide. Next, EGF (50 ng/mL) was added for 15 minutes, after which the cells were washed in chilled (4°C) PBS and the plates kept on ice. Total cell lysates were obtained and subjected to Western blot analysis as previously described (12).

The membranes were blocked for 1 hour at room temperature with 5% bovine serum albumin in 0.1% Tween 20 in tris-buffered saline (TBS-T) and incubated overnight at 4°C in anti-EGFR (Upstate Biotechnology, Inc., Lake Placid, NY; 1:500), anti-phospho-EGFR (Cell Signaling, Beverly, MA; 1:500), anti-Akt (Cell Signaling; 1:1000), anti-phospho-Akt (Cell Signaling; 1:1000), anti-Mitogen-activated protein kinase (MAPK) (Cell Signaling; 1:1000) or anti-phospho-MAPK (Cell Signaling; 1:1000) in the membrane-blocking solution described above. Next, the membranes were washed with TBS-T and incubated for 1 hour at room temperature in horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) to detect EGFR and phospho-EGFR or species-appropriate fluorescently conjugated (goat anti-rabbit IRDye 800 and goat anti-mouse IRDye 800) for another protein. Membranes were then analyzed using the SuperSignal West chemiluminescent system (Pierce Biotechnology, Rockford, IL) and an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE), and where relevant, signal intensity was determined using Odyssey software (LI-COR Biosciences). To verify equal loading of proteins, membranes were stripped and reprobed with anti- β -actin (1:5000).

Orthotopic Nude Mouse Model of HNSCC

FaDu and SCC61 cells were harvested from subconfluent cultures by trypsinization and washed with PBS. An orthotopic nude mouse model of an oral tongue tumor model was established by injecting FaDu (2×10^5) or SCC61 (1.5×10^5) cells suspended in 30 μL serum-free DMEM into the mouse tongue as described previously (14).

Fourteen to 16 days after the cells were injected, the mice were randomly assigned to 1 of 4 groups (7–9 mice per group): control, paclitaxel, vandetanib, or combination vandetanib + paclitaxel treatment. Drug regimens were administered as follows (15): the control group was given 200 μL of 1% Tween 80 by oral gavage once daily and 200 μL PBS intraperitoneally once weekly; the paclitaxel group was given 200 μL 1% Tween 80 by oral gavage once daily and 200 μg of paclitaxel (in 200 μL) intraperitoneally once weekly; the vandetanib group was given vandetanib by oral gavage at 50 mg/kg once daily and 200 μL of PBS intraperitoneally once weekly; and the combination group was given vandetanib by oral gavage at 50 mg/kg once daily and 200 μg of paclitaxel intraperitoneally, once weekly.

The mice were examined twice a week for tumor size and weight loss. Tongue tumor size was measured with microcalipers. Tumor volume was calculated as $(A)(B^2)\pi/6$, where A is the longest dimension of the tumor and B is the dimension of the tumor perpendicular to A. Mice were euthanized by CO_2 asphyxiation when they had lost more than 20% of their preinjection body weight or at 28 days post-treatment, whichever came first. Half of the tumors were fixed in formalin and embedded in paraffin for immunohistochemical and hematoxylin-and-eosin staining; the other half were embedded in optimal cutting temperature (OCT) compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at -80°C . The cervical lymph nodes were also embedded in paraffin, sectioned, stained with hematoxylin and eosin, and evaluated for metastases.

Immunohistochemical Analysis and Immunofluorescence Double Staining Analysis for CD31/Terminal Deoxynucleotidyl Transferase-mediated Deoxyuridine Triphosphate Nick-end Labeling

Frozen tissues prepared as described above were used for immunohistochemical-immunofluorescent analysis. Slides were prepared as previously described (12). Immunostaining for CD31/PECAM-1 (BD Pharmingen; 1:400) was done using the methods indicated previously (12). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was done using DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) as follows. Samples were fixed with 4% paraformaldehyde for 10 minutes, washed twice with PBS for 5 minutes, and then incubated with 0.2% Triton X-100 for 15 minutes. After two 5-minute washes with PBS, the samples were incubated with equilibration buffer for 10 minutes. At that time, the equilibration buffer was drained, and reaction buffer (44 μ L equilibration buffer, 5 μ L nucleotide mix, and 1 μ L TDT) was added to the samples and the samples were then incubated in a humid atmosphere at 37°C for 1 hour, avoiding light exposure. The reaction was terminated by immersing the samples in 2 $\times$ standard saline citrate for 15 minutes. Samples were then washed with PBS to remove unincorporated fluorescein-dUTP. For CD31-TUNEL double staining, after being fixed with acetone, the samples were washed with PBS, incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 minutes, and then incubated in a 1:400 dilution of anti-CD31/PECAM-1 antibody (BD Pharmingen) for 1 hour. After being washed with PBS, the slides samples were incubated in a 1:600 dilution of a secondary antibody conjugated with Alexa Fluor 594 (Molecular Probes) for 1 hour, avoiding light exposure. The samples were sequentially stained in the TUNEL assay, creating the CD31-TUNEL double staining.

Immunofluorescence microscopy was done using a Leica DMLA microscope (Leica Microsystems, Bannockburn, IL) equipped with a 100-watt HBO mercury bulb and filter sets (Chroma, Inc., Brattleboro, VT) to individually select red and blue fluorescent images. Images were captured using a cooled charge-coupled device Hamamatsu C5810 camera (Hamamatsu Corp., Bridgewater, NJ) and ImagePro Plus 6.0 software (Media Cybernetics, Silver Spring, MD). Photomontages were prepared using Adobe Photoshop software version 10.0.1 (Adobe Systems, Inc., San Jose, CA).

Quantification of Microvessel Density and Apoptotic Endothelial Cells

For the quantification analysis, 3 slides were prepared for each treatment group. The percentage of stained cells among the total number of cells in each field and the average proportion of stained cells in each group were calculated and compared. To quantify TUNEL expression, we counted the cells positively stained in 8 random 0.04-mm² fields at $\times$ 200 magnification per slide. To quantify microvessel density (MVD), we identified areas containing high numbers of tumor-associated blood vessels at $\times$ 100 magnification. Vessels that had stained completely with anti-CD31 antibody were counted in 8 random 0.04-mm² fields at $\times$ 200 magnification. The number of apoptotic endothelial cells was calculated as the average of the ratio of apoptotic endothelial cells to the total number of endothelial cells in 8 random 0.04-mm² fields at $\times$ 200 magnification. Specimens from the combination group (vandetanib + paclitaxel) were quantified in 8 random 0.04-mm² fields at $\times$ 200 magnification.

Statistical Analysis

Tumor volumes from the control group were compared with those from the 3 treatment groups using the two-tailed *t* tests. Survival was analyzed by the Kaplan-Meier method and compared with log-rank tests. Fisher's exact test was used to analyze associations between treatment groups and incidence of cervical lymph node metastases. Quantitative data related

to the immunohistochemical expression of CD31 and CD31-TUNEL was compared with two-tailed Student's *t* tests. Furthermore, a two-way analysis of variance was used to test for the effects of vandetanib and paclitaxel as single agents and for interaction between the compounds, as positive interaction represents drug synergy. Analysis was performed with GraphPad Prism version 5.01 (GraphPad Software). For all comparisons, $P < 0.05$ was considered statistically significant.

RESULTS

Vandetanib inhibits cell proliferation *in vitro*

To determine the effects of vandetanib on HNSCC cell proliferation *in vitro*, we first performed MTT assays with 50 HNSCC cell lines (data not shown) to obtain GI50 values for each cell line and then chose FaDu and SCC61 as relatively vandetanib-sensitive and vandetanib-resistant, respectively. Proliferation of all cell lines was inhibited in a dose-dependent manner, with GI50 values for vandetanib of 0.789 μ M for FaDu cells and 7.041 μ M for SCC61 cells (Figure 1).

Vandetanib inhibits EGFR phosphorylation *in vitro*

To determine whether vandetanib inhibits the phosphorylation of EGFR and its downstream signaling kinases in HNSCC, we treated EGF-stimulated human HNSCC cell lines FaDu and SCC61 with vandetanib. This treatment revealed that vandetanib inhibited EGF-stimulated phosphorylation of EGFR, Akt, and MAPK in a dose-dependent manner, while the total expression of the non-phosphorylated kinases remained constant (Figure 2). The concentrations of vandetanib required to inhibit the phosphorylation of downstream signaling proteins Akt and MAPK were greater in the more vandetanib-resistant SCC61 cell line than in the FaDu cell line.

Vandetanib induces apoptosis *in vitro*

To assess the effects of vandetanib on apoptosis *in vitro*, we treated FaDu and SCC61 cells with increasing concentrations of vandetanib and used PI and flow cytometry to evaluate for percentage of apoptotic cells. Vandetanib induced apoptosis in a dose-dependent manner, with FaDu (vandetanib-sensitive) cells being more sensitive than SCC61 (vandetanib-resistant) cells at 10 μ M (Figure 3).

Vandetanib, alone or in combination with paclitaxel, inhibits human HNSCC tumor growth in an orthotopic nude mouse model

We analyzed HNSCC tumor growth in an orthotopic mouse model. Figure 4 shows that the mean SCC61 tumor volumes of mice in the vandetanib group and the vandetanib and paclitaxel combination group were significantly lower than those of mice in the control group ($P=0.0310$, $P=0.0036$, respectively) on day 21 of treatment. The mean tumor volume of mice in the paclitaxel group was also lower than that of mice in the control group; however, the difference was not statistically significant ($P=0.4509$).

These antitumor effects of treatment with vandetanib and/or paclitaxel were also observed in the FaDu orthotopic model (Figure 4). The mean tumor volume was significantly lower in the combination-treated group than in the control group at all time points ($P=0.0406$); however, the differences between the control group and the paclitaxel- or vandetanib-only groups were not statistically significant ($P=0.8188$ and $P=0.3056$, respectively). Vandetanib appeared well-tolerated with no evidence of treatment-related weight loss.

Vandetanib, alone or in combination with paclitaxel, prolongs survival in an orthotopic nude mouse model of human HNSCC

We also analyzed the effect of treatment on survival in an orthotopic mouse model of HNSCC. When tumors were palpable at day 14–16 after injection, the animals were randomized to groups that received vandetanib, paclitaxel, combined vandetanib and paclitaxel, or drug vehicle. Mice were euthanized when they had lost more than 20% of their initial body weight or at the end of the study (28 days treatment).

All of the FaDu control mice in the survival study were euthanized by day 27 after cell inoculation. The median survival periods for the control, paclitaxel, vandetanib, and combination groups in the FaDu mice were 27, 34, 39, and 44 days, respectively. FaDu mice treated with vandetanib and the vandetanib and paclitaxel combination survived longer than control mice ($P=0.0019$, $P=0.0004$, log-rank test, respectively). All SCC61 mice treated with vandetanib and the vandetanib and paclitaxel combination survived until the end of the study ($P<0.0001$, $P<0.0001$ vs. control, log-rank test, respectively). The median survival periods for the control and paclitaxel-, vandetanib-, and combination-treated SCC61 mice were 26, 27, 42, and 42 days, respectively (Figure 5).

Vandetanib, alone or in combination with paclitaxel, inhibits the incidence of cervical lymph node metastases in an orthotopic nude mouse model of HNSCC

In the groups of animals with the FaDu tumors, which have metastatic potential to the cervical lymph nodes, superficial neck lymph nodes were harvested and examined histologically to determine the impact of treatment on cervical lymph node metastases. As shown in Table 1, cervical lymph node metastases were detected in 66.7% of control mice, 77.8% of paclitaxel-treated mice, 33.3% of vandetanib-treated mice, and 20% of combination-treated mice. While the difference between control group and treatment group did not reach statistical significance, both vandetanib group and the combination group had a much lower incidence of cervical lymph node metastases. In the groups of animals with SCC61 tumor, only 22.2% of control mice and 12.5% of paclitaxel-treated mice showed cervical lymph node metastases while no cervical lymph node metastasis was found in the vandetanib group and the combination group. The difference between control group and each treatment group was not significant statistically.

Vandetanib, alone or in combination with paclitaxel, increases tumor endothelial cell apoptosis and decreases MVD *in vivo*

Immunostaining of tumor sections with CD31 antibody showed that the MVD of tumors from mice treated with vandetanib (18.16 ± 1.18 , $P < 0.001$) or the vandetanib and paclitaxel combination (14.84 ± 1.36 , $P < 0.001$) was significantly lower than that of tumors in control mice (34.56 ± 1.81) (Figure 6B).

TUNEL assay showed that tumor cells in mice treated with vandetanib, alone or in combination with paclitaxel, underwent apoptosis and did so to a greater extent than tumor cells from the control group (control, $4.32\% \pm 0.58\%$; paclitaxel, 12.46 ± 2.33 , $P < 0.01$; vandetanib, 14.95 ± 1.47 , $P < 0.001$ vs. control; and vandetanib + paclitaxel, 27.31 ± 3.38 , $P < 0.0001$ vs. control) (Figure 6C). Finally, double staining for CD31-TUNEL showed that the percentage of apoptotic endothelial cells was significantly higher in the tumors of mice treated with vandetanib and the vandetanib and paclitaxel combination than in tumors of mice in the control group (control, 0%; paclitaxel, 1.24 ± 0.52 , $P < 0.05$ vs. control; vandetanib, 13.45 ± 3.20 , $P < 0.001$ vs. control; and vandetanib + paclitaxel, 17.28 ± 4.17 , $P < 0.001$ vs. control) (Figure 6D). These immunohistochemical results support the notion that vandetanib decreases MVD associated with increased apoptosis of tumor-associated endothelial cells *in vivo*.

DISCUSSION

In this study, we demonstrated that vandetanib effectively inhibits ligand-dependent phosphorylation of EGFR and its downstream mediators, Akt and MAPK. Vandetanib treatment also leads to inhibition of cell proliferation *in vitro* and *in vivo*, induction of tumor and endothelial cell apoptosis *in vivo* (alone or in combination with paclitaxel), a decrease in tumor size *in vivo*, and prolonged survival in an orthotopic nude mouse model. These biologic findings underscore the potential effect of this drug in future clinical trials for patients with HNSCC.

Several studies have reported overexpression of EGFR, a tyrosine kinase receptor that mediates several cellular functions associated with tumor progression, including proliferation, inhibition of apoptosis, and invasion (16,17). The two EGFR-targeting strategies with the best clinical outcomes are the small-molecule tyrosine kinase inhibitors and the anti-EGFR monoclonal antibodies. The efficacy of tyrosine kinase inhibitors (gefitinib and erlotinib) for recurrent and metastatic HNSCC have been studied in phase III clinical trials (18). The most widely studied anti-EGFR monoclonal antibody is cetuximab, a human-murine chimeric immunoglobulin. Cetuximab was shown to improve survival in combination with radiotherapy in locally advanced HNSCC and improve response rates in recurrent or metastatic disease when used with cytotoxic chemotherapy agents (19). Other monoclonal antibodies in early clinical trials for HNSCC include panitumumab and h-R3 (20).

Tumor growth depends on an adequate vascular supply, which is mediated by angiogenic growth factors. The most potent angiogenic factor specific to endothelial cells is VEGF (21). HNSCC growth has been shown to be associated with the presence of VEGF and upregulation of the VEGF-R in tumor endothelial cells (22). The most developed monoclonal antibody against VEGF at this time is bevacizumab which has shown synergistic effects with chemotherapy. This combination has already been approved as first-line treatment for colorectal cancer, non-small cell lung and breast cancers. Conceptually, despite the success of bevacizumab through highly selective inhibition of VEGF-A-dependent signaling, achieving therapeutic goals through multi-target therapies is a more attractive approach for heterogeneous tumors. Targeting two pathways simultaneously (i.e., inhibiting EGFR and VEGFR signaling) may also limit the development of acquired drug resistance. Studies in preclinical HNSCC models have shown that inhibiting both EGFR and VEGFR signaling can effectively inhibit tumor growth and induce apoptosis (12). Therefore, the strategy of combining antiangiogenic and chemotherapeutic agents can be applied to a broad range of tumor types, including HNSCC (10).

In the present study, we examined whether vandetanib would inhibit the growth of HNSCC cells injected into the tongues of nude mice and improve their survival. Because tyrosine kinase inhibition can enhance the antitumor activity of cytotoxic drugs (23,24), we also investigated the combined effects of vandetanib and paclitaxel on HNSCC. Paclitaxel inhibits cellular division by hyperstabilizing microtubule growth and is a commonly used chemotherapeutic agent for a variety of cancers, including HNSCC (25). Paclitaxel has also been shown to induce apoptosis in a human HNSCC cell line in a dose- and time-dependent manner (26). In addition, its antitumor activity *in vitro* has been shown to be potentiated by combination with EGFR tyrosine kinase inhibitors such as gefitinib (27).

In our study, treatment with vandetanib monotherapy significantly inhibited tumor growth and increased survival *in vivo*. Vandetanib, given by oral gavage to nude mice with FaDu tongue tumors and SCC61 tongue tumors, reduced tumor size by 42% and 53%,

respectively, compared with control mice at endpoint. Vandetanib treatment also significantly increased survival when compared with control mice.

The vandetanib and paclitaxel combination prolonged survival inhibited the tumor growth, induced apoptosis in tumor and endothelial cells, and suppressed MVD compared with vandetanib group, indicating that adding paclitaxel confers additive growth inhibition by vandetanib. The combination treatment led to 75% decreases in the mean tumor volumes in both cell lines. In addition, our quantitative immunohistochemical analysis suggests that the combination treatment of vandetanib and paclitaxel had a greater effect on the induction of tumor cell apoptosis compared with each single treatment group. The angiogenic effect of vandetanib alone was evaluated by quantifying the MVD. We found that vandetanib significantly decreased MVD which is consistent with the results of previous reports (28,29). In our study, paclitaxel decreased MVD slightly and induced slight but significant apoptosis in the endothelial cells. These data are consistent with those from previous studies, which indicated paclitaxel has antiangiogenic activity, (30,31) and they suggest that the combined effect of paclitaxel and vandetanib might be greater on endothelial cells and tumor cell apoptosis than the effect of either agent alone.

While the SCC61 cell line showed resistance against vandetanib from our MTT assay data and our apoptosis data *in vitro* compared with Fadu cell line, we found no major differences in the *in vivo* antitumor effects of vandetanib between FaDu tumors and SCC61 tumors. This might be because the antitumor effects of vandetanib in HNSCC may result primarily from inhibiting VEGF signaling and, thus, represent indirect antitumor effects rather than direct antiproliferative effects on the tumor. These findings might reflect the biological differences between the two cell lines.

While the difference was not significant statistically, the combination group had a much lower incidence of cervical lymph node metastases, with only one mouse having cervical lymph node metastasis. This finding is consistent with our previous report in which combined anti-EGFR and VEGFR-2 antibody targeted therapy using DC101 and cetuximab inhibited the growth of SCC of the oral tongue and also decreased the incidence of the neck lymph node metastases on orthotopic xenografts of metastatic squamous cell carcinoma of the oral tongue (32). As angiogenesis is thought to play an important role in tumor growth and metastasis (33) and the EGFR pathway is one of the major growth and survival signaling pathways in HNSCC, blocking these two pathways could be very helpful in the treatment of cervical nodal disease in HNSCC patients.

Preclinical studies of vandetanib in gastric, pancreatic, prostate, and colon cancer orthotopic tumor models have shown findings that support our findings with regard to its potential antitumorigenic and survival-prolonging effects (29,34–36). Several papers have reported the impact of treatment of HNSCC with vandetanib (37,38), however, this is the first report that assessed the effects of treatment with vandetanib, alone and in combination with paclitaxel in an orthotopic mouse model of HNSCC. The oral bioavailability of vandetanib makes it more convenient for use than monoclonal antibodies. Based on successful preclinical results, clinical trials of vandetanib have advanced quickly and have shown promise. A recent clinical study reported that the combination treatment with vandetanib (100 mg) and docetaxel in patients with stage III or stage IV non-small cell lung cancer increased progression-free survival significantly compared to docetaxel treatment alone (39). Clinical trials of vandetanib for HNSCC are also under way. A phase I study of vandetanib combined with radiation therapy in patients with previously untreated, unresected, stage III or stage IV HNSCC reported that 100 mg of vandetanib was the maximum tolerated dose with radiation therapy and cisplatin treatment (40). Further clinical studies of vandetanib for HNSCC are needed.

In summary, we have corroborated previous reports of targeted *in vitro* EGFR and VEGFR-2 tyrosine kinase inhibition by vandetanib (9). Our findings also demonstrate the pre-clinical efficacy of vandetanib, as a dual inhibitor of EGFR and VEGFR-2 signaling, in diminishing *in vivo* tumor growth and prolonging survival in mice with orthotopic human HNSCC xenografts. These favorable results support the evaluation of vandetanib in clinical trials, some of which have been initiated at our center, for patients with HNSCC.

Acknowledgments

We thank Markeda Wade for her critical editorial review of the manuscript. ZACTIMATM is a registered trademark of the AstraZeneca group of companies.

Grant support: This work was supported by AstraZeneca, an American Society of Clinical Oncology Young Investigator Award (P.K. Morrow), the U.T. M.D. Anderson Cancer Center PANTHEON program, NIH Specialized Program of Research Excellence Grant P50CA097007, National Research Science Award Institutional Research Training Grant T32CA60374 and NIH Cancer Center Support (Core) GrantCA016672.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics. *CA Cancer J Clin*. 2009; 59(4):225–49. [PubMed: 19474385]
2. Goldberg HI, Lockwood SA, Wyatt SW, Crossett LS. Trends and differentials in mortality from cancers of the oral cavity and pharynx in the United States, 1973–1987. *Cancer*. 1994; 74(2):565–72. [PubMed: 8033034]
3. Baselga J. Targeting tyrosine kinases in cancer: the second wave. *Science*. 2006; 312(5777):1175–8. [PubMed: 16728632]
4. Cantley LC, Auger KR, Carpenter C, et al. Oncogenes and signal transduction. *Cell*. 1991; 64(2):281–302. [PubMed: 1846320]
5. Thomas GR, Nadiminti H, Regalado J. Molecular predictors of clinical outcome in patients with head and neck squamous cell carcinoma. *Int J Exp Pathol*. 2005; 86(6):347–63. [PubMed: 16309541]
6. Artese L, Rubini C, Ferrero G, Fioroni M, Santinelli A, Piattelli A. Microvessel density (MVD) and vascular endothelial growth factor expression (VEGF) in human oral squamous cell carcinoma. *Anticancer Res*. 2001; 21(1B):689–95. [PubMed: 11299827]
7. Mineta H, Miura K, Ogino T, Takebayashi S, Misawa K, Ueda Y. Vascular endothelial growth factor (VEGF) expression correlates with p53 and ki-67 expressions in tongue squamous cell carcinoma. *Anticancer Res*. 2002; 22(2B):1039–44. [PubMed: 12168898]
8. Oc P, Rhys-Evans P, Eccles SA. Expression of vascular endothelial growth factor family members in head and neck squamous cell carcinoma correlates with lymph node metastasis. *Cancer*. 2001; 92(3):556–68. [PubMed: 11505400]
9. Ryan AJ, Wedge SR. ZD6474—a novel inhibitor of VEGFR and EGFR tyrosine kinase activity. *Br J Cancer*. 2005; 92 (Suppl 1):S6–13. [PubMed: 15928657]
10. Kerbel RS. Antiangiogenic therapy: a universal chemosensitization strategy for cancer? *Science*. 2006; 312(5777):1171–5. [PubMed: 16728631]
11. Weichselbaum RR, Dahlberg W, Beckett M, et al. Radiation-resistant and repair-proficient human tumor cells may be associated with radiotherapy failure in head- and neck-cancer patients. *Proc Natl Acad Sci U S A*. 1986; 83(8):2684–8. [PubMed: 3458227]
12. Yigitbasi OG, Younes MN, Doan D, et al. Tumor cell and endothelial cell therapy of oral cancer by dual tyrosine kinase receptor blockade. *Cancer Res*. 2004; 64(21):7977–84. [PubMed: 15520205]
13. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods*. 1991; 139(2):271–9. [PubMed: 1710634]
14. Myers JN, Holsinger FC, Jasser SA, Bekele BN, Fidler IJ. An orthotopic nude mouse model of oral tongue squamous cell carcinoma. *Clin Cancer Res*. 2002; 8(1):293–8. [PubMed: 11801572]

15. Ciardiello F, Caputo R, Damiano V, et al. Antitumor effects of ZD6474, a small molecule vascular endothelial growth factor receptor tyrosine kinase inhibitor, with additional activity against epidermal growth factor receptor tyrosine kinase. *Clin Cancer Res*. 2003; 9(4):1546–56. [PubMed: 12684431]
16. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. *Cell*. 1990; 61(2):203–12. [PubMed: 2158859]
17. Wells A. EGF receptor. *Int J Biochem Cell Biol*. 1999; 31(6):637–43. [PubMed: 10404636]
18. Stewart JS, Cohen EE, Licitra L, et al. Phase III study of gefitinib compared with intravenous methotrexate for recurrent squamous cell carcinoma of the head and neck [corrected]. *J Clin Oncol*. 2009; 27(11):1864–71. [PubMed: 19289630]
19. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med*. 2006; 354(6):567–78. [PubMed: 16467544]
20. Cohen EE. Role of epidermal growth factor receptor pathway-targeted therapy in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck. *J Clin Oncol*. 2006; 24(17):2659–65. [PubMed: 16763280]
21. Jain RK. Molecular regulation of vessel maturation. *Nat Med*. 2003; 9(6):685–93. [PubMed: 12778167]
22. Baillie R, Harada K, Carlile J, Macluskey M, Schor SL, Schor AM. Expression of vascular endothelial growth factor in normal and tumour oral tissues assessed with different antibodies. *Histochem J*. 2001; 33(5):287–94. [PubMed: 11563542]
23. Kim SJ, Uehara H, Yazici S, et al. Simultaneous blockade of platelet-derived growth factor-receptor and epidermal growth factor-receptor signaling and systemic administration of paclitaxel as therapy for human prostate cancer metastasis in bone of nude mice. *Cancer Res*. 2004; 64(12):4201–8. [PubMed: 15205332]
24. Morelli MP, Cascone T, Troiani T, et al. Sequence-dependent antiproliferative effects of cytotoxic drugs and epidermal growth factor receptor inhibitors. *Ann Oncol*. 2005; 16(Suppl 4):iv61–68. [PubMed: 15923432]
25. Ozer E, Grecula JC, Agrawal A, Rhoades CA, Young DC, Schuller DE. Long-term results of a multimodal intensification regimen for previously untreated advanced resectable squamous cell cancer of the oral cavity, oropharynx, or hypopharynx. *Laryngoscope*. 2006; 116(4):607–12. [PubMed: 16585867]
26. Nonaka M, Ikeda H, Fujisawa A, Uehara M, Inokuchi T. Induction of apoptosis by paclitaxel in human oral carcinoma cells. *Int J Oral Maxillofac Surg*. 2006; 35(7):649–52. [PubMed: 16513327]
27. Ciardiello F, Caputo R, Bianco R, et al. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res*. 2000; 6(5):2053–63. [PubMed: 10815932]
28. Wu W, Onn A, Isobe T, et al. Targeted therapy of orthotopic human lung cancer by combined vascular endothelial growth factor and epidermal growth factor receptor signaling blockade. *Mol Cancer Ther*. 2007; 6(2):471–83. [PubMed: 17308046]
29. McCarty MF, Wey J, Stoeltzing O, et al. ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor with additional activity against epidermal growth factor receptor tyrosine kinase, inhibits orthotopic growth and angiogenesis of gastric cancer. *Mol Cancer Ther*. 2004; 3(9):1041–8. [PubMed: 15367698]
30. Grant DS, Williams TL, Zahaczewsky M, Dicker AP. Comparison of antiangiogenic activities using paclitaxel (taxol) and docetaxel (taxotere). *Int J Cancer*. 2003; 104(1):121–9. [PubMed: 12532428]
31. Muta M, Yanagawa T, Sai Y, et al. Effect of low-dose Paclitaxel and docetaxel on endothelial progenitor cells. *Oncology*. 2009; 77(3–4):182–91. [PubMed: 19729975]
32. Sano D, Choi S, Milas ZL, et al. The effect of combination anti-endothelial growth factor receptor and anti-vascular endothelial growth factor receptor 2 targeted therapy on lymph node metastasis: a study in an orthotopic nude mouse model of squamous cell carcinoma of the oral tongue. *Arch Otolaryngol Head Neck Surg*. 2009; 135(4):411–20. [PubMed: 19380367]
33. Kerbel RS. Tumor angiogenesis: past, present and the near future. *Carcinogenesis*. 2000; 21(3):505–15. [PubMed: 10688871]

34. Hammarsten P, Halin S, Wikstom P, Henriksson R, Rudolfsson SH, Bergh A. Inhibitory effects of castration in an orthotopic model of androgen-independent prostate cancer can be mimicked and enhanced by angiogenesis inhibition. *Clin Cancer Res.* 2006; 12(24):7431–6. [PubMed: 17189416]
35. Conrad C, Ischenko I, Kohl G, et al. Antiangiogenic and antitumor activity of a novel vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor ZD6474 in a metastatic human pancreatic tumor model. *Anticancer Drugs.* 2007; 18(5):569–79. [PubMed: 17414626]
36. Troiani T, Serkova NJ, Gustafson DL, et al. Investigation of two dosing schedules of vandetanib (ZD6474), an inhibitor of vascular endothelial growth factor receptor and epidermal growth factor receptor signaling, in combination with irinotecan in a human colon cancer xenograft model. *Clin Cancer Res.* 2007; 13(21):6450–8. [PubMed: 17975157]
37. Sano D, Kawakami M, Fujita K, et al. Antitumor effects of ZD6474 on head and neck squamous cell carcinoma. *Oncol Rep.* 2007; 17(2):289–95. [PubMed: 17203163]
38. Gustafson DL, Frederick B, Merz AL, Raben D. Dose scheduling of the dual VEGFR and EGFR tyrosine kinase inhibitor vandetanib (ZD6474, Zactima) in combination with radiotherapy in EGFR-positive and EGFR-null human head and neck tumor xenografts. *Cancer Chemother Pharmacol.* 2008; 61(2):179–88. [PubMed: 17393165]
39. Herbst RS, Sun Y, Korf S, et al. Vandetanib plus docetaxel versus docetaxel as second-line treatment for patients with advanced non-small cell lung cancer (NSCLC): A randomized, double-blind phase III trial (ZODIAC). *J Clin Oncol.* 2009; 27(18s) abstr CRA8003.
40. Papadimitrakopoulou V, Frank SJ, Blumenschein GR, et al. Phase I evaluation of vandetanib with radiation therapy (RT) ± cisplatin in previously untreated advanced head and neck squamous cell carcinoma (HNSCC). *J Clin Oncol.* 2009; 27(15S) abstr 6016.

Figure 1.

Antiproliferative effects of vandetanib on the human HNSCC cell lines FaDu, and SCC61. Cells were cultured in 96-well plates, treated with the indicated concentrations of vandetanib, and tested for proliferation with an MTT assay. GI_{50} values were $0.789 \mu M$ for FaDu, and $7.041 \mu M$ for SCC61. Points indicate means of triplicate experiments; bars, SD.

Figure 2.

Dose-dependent inhibition of EGFR phosphorylation by vandetanib in the human HNSCC cell lines FaDu, and SCC61. Cells were serum-starved, treated for 90 min with vandetanib at the indicated concentrations, and then stimulated for 15 min with 50 ng/ml EGF. Whole-cell lysates were obtained and subjected to Western immunoblotting to resolve proteins. Antibodies to total (unphosphorylated) receptors and β -actin were used as protein loading controls.

Figure 3.

In vitro effects of vandetanib on apoptosis induction in human HNSCC cells. Cultured HNSCC cells were treated with 0–10 μM vandetanib and the percentages of apoptotic cells determined by flow cytometry. Columns indicate means of triplicate experiments; bars, SD.

Figure 4.

In vivo effects of vandetanib on tumor growth in mice. FaDu and SCC61 human HNSCC cells were injected into the tongues of nude mice. After tumor nodules had developed, mice were treated by oral gavage with vandetanib at 50 mg/kg daily, intraperitoneally with paclitaxel at 200 μ g, with a combination of vandetanib and paclitaxel at the same doses, or with vehicle alone (control group). Tumors were measured with microcalipers twice a week. Points indicate mean tumor volumes, in mm^3 ; bars, SE; *, $P < 0.05$ as compared with controls; **, $P < 0.01$ as compared with controls.

Figure 5.

In vivo effects of vandetanib on survival time in mice. FaDu and SCC61 cells were injected into the tongues of nude mice. After tumor nodules developed, mice were treated by oral gavage with vandetanib at 50 mg/kg daily, intraperitoneally with paclitaxel at 200 μ g, with a combination of vandetanib and paclitaxel at the same doses, or with vehicle alone (control group). Subject animals were euthanized when they had lost more than 20% of their initial body weight or after 28 days of treatment. Survival was analyzed by the Kaplan-Meier method and compared with log-rank tests.

Figure 6.

Immunohistochemical analyses of FaDu xenograft tumors in nude mice. Tumors were harvested after 10 days of treatment, and representative sections obtained from FaDu tumors were immunostained for expression of CD31 (endothelial cell marker) and TUNEL (tumor cell apoptosis). Magnification, $\times 200$. Double staining for CD31 (red)-TUNEL (green) was also performed to reveal induction of apoptosis in tumor-associated endothelial cells. Magnification, $\times 400$. Results of quantitative analysis for CD31 staining (microvessel density) (B), TUNEL staining (C), and endothelial cells apoptosis (D). *Columns*, mean; *bars*, SE; *, $P < 0.05$; **, $P < 0.01$ as compared with controls; ***, $P < 0.001$ as compared with controls.

Table 1

Effects of vandetanib and paclitaxel on the incidence of lymph node metastasis in nude mice bearing orthotopic HNSCC xenografts. The lymph node metastases rates on FaDu mice were 66.7%, 77.8%, 33.3% and 20.0% for the control, paclitaxel, vandetanib and combination groups respectively. The lymph node metastases rates on SCC61 mice were 22.2%, 12.5%, 0.0% and 0.0% for the control, paclitaxel, vandetanib and combination groups respectively. The difference between control group and each treatment group did not reach statistical significance.

Treatment	% Mice with cervical lymphatic metastasis	
	FaDu (n=7-9)	SCC61 (n=8-9)
control	66.7%	22.2%
paclitaxel	77.8%	12.5%
vandetanib	33.3%	0.0%
vandetanib + paclitaxel	20.0%	0.0%