Vandetanib Inhibits Growth of Adenoid Cystic Carcinoma in an Orthotopic Nude Mouse Model

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Abstract Purpose: Adenoid cystic carcinoma (ACC) can often be controlled with surgery and postoperative adjuvant radiotherapy but is also characterized by late local recurrence and distant metastasis. No effective systemic therapeutic agents have been found to alter the natural history of ACC. Therefore, new therapeutic approaches are needed. In this study, we evaluated whether vandetanib (Zactima), a potent inhibitor of vascular endothelial growth factor receptor-2 (VEGFR-2) and epidermal growth factor receptor (EGFR) tyrosine kinases, had antitumor efficacy in vitro and in vivo in an orthotopic nude mouse model of human ACC. Experimental Design: The in vitro effects of vandetanib were assessed in three ACC cell lines on cell growth, apoptosis, and VEGFR-2 and EGFR phosphorylation levels. The in vivo antitumor activity of vandetanib was examined in nude mice bearing parotid gland ACC tumors. The mice were treated for 4 weeks with vandetanib (50 mg/kg/d) or placebo (control). Tumors were resected at necropsy, and immunohistochemical and immunofluorescence staining were done. Results: In vitro, vandetanib caused dose-dependent inhibition of VEGFR-2 and EGFR phosphorylation in ACC cells. Vandetanib also inhibited the cell proliferation and induced their dose-dependent apoptosis. In vivo, mice in the vandetanib group had tumor volumes significantly lower than those in the control group (P < 0.01). In addition, immunohistochemical staining showed a decrease in microvessel density and an increase in apoptosis of both tumor cells and endothelial cells within the tumor xenografts. Conclusion: These results suggest that vandetanib inhibits the growth of ACC in vitro and in vivo, making it a promising novel agent for the treatment of ACC.

Adenoid cystic carcinoma (ACC) is one of the most common types of salivary gland cancers, accounting for ~22% of salivary gland malignancies and ~1% of all head and neck cancers (1, 2). ACC is a slow-growing tumor with an often protracted clinical course; some patients with ACC can live for 10 to 20 years after distant metastasis is diagnosed (3). Despite the relatively favorable 5-year survival rates for patients with ACC (50-90%), the disease-specific survival rates at 10 and 25 years are lower (40% and 15%, respectively; ref. 4). The poor long-term prognosis for patients with ACC is mainly due to local recurrence related to perineural invasion and delayed onset of distant metastasis (5). The primary treatment for ACC is surgery followed by postoperative radiation therapy because of the high risk of local recurrence rates (6, 7). Chemotherapy is used to manage recurrent ACC that is no longer amenable to additional surgery or radiation therapy and to palliate symptoms from distant metastases. However, radiation therapy and chemotherapy have proven to be ineffective for locally recurrent and distant ACC; in fact, the response of ACC to systemic therapy is modest at best (1). To date, no standard treatment has been devised that inhibits the progression of ACC tumors and improves survival from this disease. A better understanding of the biology of ACC potentially would permit the development of therapeutic approaches based on biologically relevant targets.

Numerous preclinical and clinical studies on molecular targeted therapy have shown that this therapeutic approach has great promise in the treatment of various malignant tumors (8). Molecular targeted therapy is designed to inhibit key signaling pathways involved in tumor growth and metastasis. Two key molecular targets are vascular endothelial growth factor receptors (VEGFR) and the epidermal growth factor receptor (EGFR). VEGF plays a key role in tumor angiogenesis, including the induction of endothelial cell proliferation,
migration, survival, and capillary tube formation (9, 10). A recent study reported that VEGF is a major factor in angiogenesis and that microvesSEL density (MVD) is considered to be a prognostic factor for distant metastasis in ACC (11). In a previous study, we observed that VEGF/VEGFR-2 is overexpressed in human ACC tissues (12). Similarly, it has been reported in numerous studies that EGFR, a tyrosine kinase receptor, plays a critical role in tumor cell proliferation, invasion, metastasis, and survival (13, 14). In addition, EGFR is overexpressed in a variety of epithelial tumors (15). In ACC tissues, EGFR expression has been reported to range from 10% to 85% (13, 16).

The VEGF and EGFR signaling pathways seem to be closely related, particularly with respect to angiogenesis, in many tumors. The EGFR pathway increases angiogenesis by up-regulating VEGF or other key mediators in the angiogenic process (17). Activation of VEGF expression is considered to be one mechanism used by tumors to become resistant to anti-EGFR therapy (18). Moreover, preclinical studies have shown that EGFR blockade with monoclonal antibodies or small-molecule tyrosine kinase inhibitors results in down-regulation of proangiogenic mediators, including VEGF, interleukin-8, and basic fibroblast growth factor (19). Therefore, dual inhibition of the VEGF and EGFR signaling pathways theoretically can result in greater antitumor effects than inhibition of either one alone. Several biological agents that target both the VEGFR and EGFR signaling pathways have shown clinical benefit in several human cancers when administered either alone or in combination with standard cytotoxic therapies (19).

Vandetanib (Zactima, AstraZeneca Pharmaceuticals) is a small-molecule inhibitor of the VEGFR-2 and EGFR tyrosine kinase activities (20). Vandetanib has shown significant antitumor activity in various xenograft models of human cancer (21). Based on these favorable preclinical results, clinical trials of vandetanib are currently under way in several different human cancers when administered either alone or in combination with standard cytotoxic therapies (19).

In this study, we hypothesized that VEGF-2 signaling pathways might be important for the progression and metastasis of human salivary ACC and that inhibition of these pathways might be a beneficial part of an integrated treatment strategy for ACC. To test our hypothesis, we investigated the preclinical efficacy of vandetanib in an orthotopic nude mouse model of human ACC.

Materials and Methods

Cell lines and culture conditions. Human salivary ACC cell lines, ACC2, ACC3, and ACCM, were provided by the Ninth People’s Hospital (Department of Oral and Maxillofacial Surgery, Shanghai Jiao Tong University School of Medicine, Shanghai, People’s Republic of China; refs. 22, 23). The cells were maintained as monolayer cultures in RPMI 1640 containing penicillin-streptomycin (Flow Laboratories, China; refs. 22, 23). The cells were maintained as monolayer cultures in RPMI 1640 containing penicillin-streptomycin (Flow Laboratories, China; refs. 22, 23).

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Cell Signaling), anti-VEGFR-2 (Cell Signaling), anti-phospho-VEGFR-2 (Tyr1054; Biosource International), anti-Akt (Cell Signaling), anti-phospho-Akt (Ser473; Cell Signaling), anti-MAPK (Cell Signaling), and anti-phospho-MAPK (Thr202/Tyr204; Cell Signaling). Signals were visualized using the UltraSignal West Pico Chemiluminescent system (Pierce Biotechnology).

Measurement of cell proliferation. The antiproliferative ability of vandetanib against ACC2, ACC3, and ACCM cells in vitro was determined using the tetrazolium-based colorimetric (MTT) assay. Specifically, 1.5 × 10⁴ cells were plated in a 24-well plate and allowed to attach for 24 h. After 24 h, cells were treated with different concentrations of vandetanib (0–10 μmol/L) in RPMI 1640 containing 2% FBS and incubated for 72 h with vandetanib. After 72-h incubation, the cells were incubated for 2 h in medium containing MTT and then lysed in DMSO. The amount of MTT converted to formazan by the cells was measured using a microtiter plate reader at an absorbance of 570 nm (Dynatech Laboratories).

Measurement of cell death. The ACC2, ACC3, and ACCM cells were plated at a density of 2 × 10⁵ per well in six-well plates (Costar) and incubated in 2% FBS-containing medium overnight. The next day, cells were washed twice with PBS and maintained in 2% FBS-containing medium before treatment with vandetanib. After 72 h, PI staining of hypodiploid DNA was used to determine the extent of cell death. For the PI staining, the treated cells were resuspended in Nicoletti buffer (50 μg/mL PI, 0.1% sodium citrate, 0.1% Triton X-100, and 1 mg/mL RNase A in PBS) for 20 min at 4°C. Cells were analyzed by flow cytometry, and the fraction of cells with sub-G₀-G₁ DNA content was measured using the Lysis program (Becton Dickinson). The percentage of cells undergoing specific apoptosis was calculated by subtracting the percentage of cells that had undergone spontaneous apoptosis in the relevant controls from the total percentage of apoptotic cells in the study cultures.

Treatment of established human salivary carcinomas growing in the parotid gland of nude mice. Ten days after injection of the ACC3 and ACCM cells, tumor nodules were palpable. Mice with similarly sized tumors were randomized into one of two groups (n = 10 for each group): group 1, the control group, received 200 μL of 1% Tween 80 by way of oral gavage once per day as a placebo; group 2, the vandetanib group, received vandetanib by oral gavage at 50 mg/kg/d. Treatments continued for 4 wk. Tumor volume and body weight were measured twice per week. Tumor volume was measured using the formula \( V = \frac{1}{2}AB^2 \pi / 6 \), where \( A \) is the longest diameter and \( B \) is the shortest diameter of the tumor.

The mice were euthanized by means of CO₂ asphyxiation after 4 wk or earlier if they lost >20% of their preinjection body weight or became moribund. The mice were then necropsied; parotid tumors, cervical lymph nodes, and lungs were excised. For immunohistochemical and routine H&E staining, one part of the tumor tissue was fixed in formalin and embedded in paraffin. Another part was embedded in OCT compound (Miles, Inc.), rapidly frozen in liquid nitrogen, and stored at -80°C. The cervical lymph nodes and lungs were also fixed in formalin and embedded in paraffin for histologic review and determination of metastasis.

Immunohistochemical-immunofluorescence analysis of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays and CD31/PECAM-1. Immunohistochemistry was done with the anti-CD31/PECAM-1 and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining and a commercial detection kit. For staining with antibodies against CD31/PECAM-1, frozen tumors were sectioned (8- to 10-mm-thick sections), mounted on positively charged Superfrost slides (Fisher Scientific), air dried for 30 min, and fixed in cold acetone for 10 min. After fixation, the slides were washed thrice with PBS, incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min at room temperature, and then incubated with a 1:400 dilution of anti-CD31/PECAM-1 primary antibody (PharMingen) for 1 h at room temperature. The samples were then washed thrice for 3 min and blocked with protein-blocking solution for 10 min. The samples were then incubated with a 1:400 dilution of peroxidase-conjugated anti-CD31/PECAM-1 secondary antibody for 1 h at room temperature. A positive reaction was visualized by incubating the slides with diaminobenzidine (Research Genetics). Samples were then washed thrice with PBS and then counterstained with hematoxylin for 14 to 20 s at room temperature. The slides were given a final wash and then mounted with Universal Mount (Research Genetics).

To assess the intratumoral apoptosis, TUNEL assay was done using a commercial Apoptosis Detection kit (Promega). The tissues were fixed with 4% paraformaldehyde (medtech-free) for 10 min at room temperature, washed twice with PBS for 5 min, and then incubated with 0.2% Triton X-100 for 15 min at room temperature. The tissue sections were then incubated with reaction buffer containing 44 μL of equilibration buffer, 5 μL of nucleotide mix, and 1 μL of terminal deoxynucleotidyl transferase (Promega kit) at 37°C for 1 h, avoiding exposure to light. The reaction was terminated by immersing the samples in 2 × SSC for 15 min. Samples were then washed thrice for 5 min with PBS to remove unincorporated fluorescein-dUTP.

Immunofluorescence double staining for CD31/VEGFR-2, CD31/phospho-VEGFR-2, CD31/EGFR, CD31/phospho-EGFR, and CD31/TUNEL. For double immunofluorescence staining, frozen tissues were used. After fixation 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 min, and then incubated with a 1:400 dilution of rat anti-mouse CD31 monoclonal antibody (PharMingen) for 1 h at room temperature. After washing with PBS, the slides were incubated with a 1:400 dilution of secondary goat anti-rat antibody conjugated to Alexa Fluor 594 (Molecular Probes) for 1 h in the dark. These same samples were subsequently stained with TUNEL assay for double staining of CD31/TUNEL as described above. They were also sequentially stained with VEGFR-2 antibody, phospho-VEGFR-2 antibody, EGFR antibody, and phospho-EGFR antibody for double staining (CD31/VEGFR-2, CD31/phospho-VEGFR-2, CD31/EGFR, CD31/phospho-EGFR, and CD31/TUNEL), respectively as previously described (24).

Immunofluorescence microscopy was done using a Leica Axioplan2 microscope (Carl Zeiss, Inc.) equipped with a color-chilled three-chip charge-coupled device camera (model C5810; Hamamatsu Photonics) and digitized using image analysis software (Optimas). Imaging of the stained sections was done using a Microphot-FX microscope (Nikon) equipped with a three-chip charge-coupled device color video camera (model DXC990; Sony Corp.). For quantitative analysis of CD31, the tumor-associated microvessels that were completely stained were counted in 10 random 0.159-mm² fields at ×100 magnification per slide; four slides were prepared for each study group. To quantify TUNEL staining and CD31/TUNEL, we counted the positively stained cells in five random 0.039-mm² fields at ×200 magnification per slide from a total of four slides that were prepared for each study group. The photomontages were prepared using Photoshop (version 7.0; Adobe Systems, Inc.) and quantification was done using the Image-Pro Plus software package (Media Cybernetics, Inc.). To quantify TUNEL expression, the percentage of positively stained cells among the total number of cells was calculated. Quantification of apoptotic endothelial cells (CD31/TUNEL) was measured as the average of the ratio of apoptotic endothelial cells to the total number of endothelial cells.

Statistical analysis. Tumor volumes from the control group were compared with those from the three treatment groups using the paired Student’s t test to determine whether differences between them were significant at \( P < 0.01 \). The quantities of MVD, TUNEL, and CD31/TUNEL were compared using the Wilcoxon rank-sum test, with significance at \( P < 0.01 \). The Statistical Package for the Social Sciences for Windows software (version 12.0; SPSS, Inc.) was used to do statistical analysis.

Results

Vandetanib inhibits phosphorylation of the EGFR and VEGFR-2 and downstream signaling kinases in ACC cell lines. To investigate the expression levels of EGFR, VEGFR-2, and the downstream targets of VEGFR-2, Akt, and MAPK in human ACC cell lines, we did Western blot analysis of these receptors in the ACC2, ACC3, and ACCM cell lines in 10% FBS medium. We found that EGFR was expressed in all the ACC cells, with the highest level of EGFR expression observed in the ACC3 cell line. ACC3 cells also showed high levels of VEGFR-2 (Fig. 1A).

To confirm that vandetanib inhibits the phosphorylation of EGFR and VEGFR-2, Western blot analysis was done in ligand-stimulated ACC3 cells, which showed high phosphorylation levels for EGFR and VEGFR-2. Serum-starved ACC3 cells were treated with various concentrations of vandetanib for 90 min followed by stimulation with EGF for 15 min. We found that vandetanib inhibited the EGF-stimulated phosphorylation of EGFR, Akt, and MAPK in a dose-dependent manner (Fig. 1B). We also found that vandetanib inhibited the VEGF-stimulated phosphorylation of VEGFR-2 in ACC3 cells in a dose-dependent manner, with the level of inhibition attaining significance at a vandetanib concentration of 1 μmol/L (Fig. 1B).

Vandetanib inhibits proliferation and induces apoptosis in ACC cells in vitro. MTT assay revealed that ACC cell proliferation was inhibited in all cell lines in a dose-dependent fashion (Fig. 2A). The IC_{50} values of vandetanib were 4 μmol/L for ACC3, 6.7 μmol/L for ACC2, and 6.6 μmol/L for ACCM.

PI staining and flow cytometric analysis revealed that vandetanib induced apoptosis in the ACC cell lines in a dose-dependent manner. In addition, ACC3 cells were more sensitive to vandetanib than ACC2 and ACCM cells at all doses. In ACC3 cells, 50% induction of apoptosis occurred at 9.6 μmol/L (Fig. 2B), whereas in ACC2 and ACCM cells, 50% induction of apoptosis occurred at 12.7 and 17.2 μmol/L of vandetanib, respectively (data not shown).

Vandetanib inhibits the growth of ACC in an orthotopic nude mouse model of ACC. To determine the in vivo antitumor effects of vandetanib, we established ACC3 tumors in the parotid glands of mice and measured the tumor out to 4 weeks of treatment. As shown in Fig. 3A, the mean tumor volumes of mice in the vandetanib group were significantly lower than those of mice in the control group (90% decrease; \( P < 0.01 \)) on the last day of treatment. During the treatment period, vandetanib was well tolerated by the mice (i.e., we saw no adverse side effects or loss in body weight).

We confirmed the antitumor effects of vandetanib using an orthotopic nude mouse model of ACC generated by the ACCM cell line. Vandetanib significantly inhibited the tumor growth in orthotopically implanted ACCM (87% decrease; \( P < 0.01 \)) as shown in Fig. 3B.

Vandetanib decreases MVD and induces apoptosis of tumor cells and tumor-associated endothelial cells. To clarify the mechanism of the antitumor action of vandetanib, we stained tumor sections with CD31-specific antibodies and determined the MVD by measuring the number of CD31-positive cells (Fig. 4A). As shown in Table 1, the tumors of mice treated with vandetanib showed significantly lower MVD than those of mice in the control group (\( P < 0.01 \)). Compared with the controls, mice with ACC3 tumor and mice with ACCM treated with vandetanib had significantly lower tumor MVD values at 82% and 73%, respectively.

In the TUNEL staining assays (Fig. 4A), the percentages of TUNEL-positive cells in ACC3 tumors from mice in the vandetanib group and ACCM tumors from mice in the vandetanib group were 23.4 ± 2.2 and 20.4 ± 0.8, respectively (Table 1), versus 4.1% and 3.7% in placebo-treated tumors. Thus, vandetanib was responsible for a significant induction of apoptosis in these two groups relative to the control groups (\( P < 0.01 \)).

CD31/TUNEL double staining in tumor specimens from the control mice did not show any endothelial cell apoptosis (Fig. 4B); however, the percentage of apoptotic endothelial cells was significantly higher in ACC3 tumor sections from mice in the vandetanib than in control tumors (\( P < 0.01 \); Table 1). The ACCM tumors from mice in the vandetanib group also had the
apoptotic endothelial cells; however, the difference did not reach statistical significance (Table 1).

To confirm that vandetanib inhibited tumor cell proliferation by acting on the EGFR and VEGFR-2 signaling pathway in vivo, we analyzed all tumor sections for proliferating cell nuclear antigen. There were no detectable changes in proliferating cell nuclear antigen staining in response to vandetanib treatment (data not shown).

Vandetanib suppresses angiogenesis by inhibiting EGFR and VEGF phosphorylation in vivo. Double staining for CD31/EGFR, CD31/phospho-EGFR, CD31/VEGFR-2, and CD31/phospho-VEGFR-2, which was carried out with CD31 (red staining) and EGFR, phospho-EGFR, VEGFR-2, and phospho-VEGFR-2 (green staining). Although the level of expression of total EGFR and VEGFR-2 did not vary significantly among tumors from mice in two groups, tumors from the mice with vandetanib had significantly decreased phosphorylation of these two receptor kinases (Fig. 5).

Discussion

In the present study, we tested the antitumor effects of vandetanib in vitro and in an orthotopic xenograft model of ACC. Our study shows that vandetanib induces apoptosis and decreases the growth of human ACC cell lines in vitro by inhibiting the EGFR and VEGFR-2 signaling pathways. In addition, vandetanib inhibits tumor growth in an orthotopic nude mice model of parotid ACC by decreasing tumor-associated angiogenesis and inducing the apoptosis of tumor and endothelial cells.

ACC is characterized by local recurrence, late distant metastasis, and poor response to conventional chemotherapy. Therefore, new therapeutic approaches, possibly including molecular targeted therapies, are urgently needed to benefit patients with recurrent and/or metastatic ACC. Current molecular targets in ACC include c-KIT, EGFR, the human epidermal growth receptor-2, and estrogen and progesterone receptors (25). Based on those findings, trials of targeted therapy have been initiated in patients with advanced ACC.
Imatinib, which inhibits the c-KIT tyrosine kinase, has been evaluated in ACC, but no objective responses have been observed (1). The efficacy of gefitinib, trastuzumab, and lapatinib has also been investigated in salivary gland carcinomas including ACC (26–28). However, the effectiveness of these agents against ACC has also been disappointing. Therefore, other molecular targeted therapies based on additional and/or alternate molecular targets are needed, as there are currently no standard systemic therapies shown to have benefit for those afflicted with recurrent and/or metastatic ACC.

Tumors require new vasculature to grow, and angiogenesis is crucial for the progression and metastasis of many types of human tumors (29). Most cancer deaths result from metastases, making metastatic lesions an important target of antiangiogenic

Table 1. Quantitative analysis of immunohistochemical staining of ACC tumors in parotid glands of nude mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment group (ACC3)</th>
<th>Treatment group (ACCM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Vandetanib</td>
</tr>
<tr>
<td>Tumor cells (mean ± SE)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUNEL (%)*</td>
<td>4.1 ± 0.7</td>
<td>23.4 ± 2.2†</td>
</tr>
<tr>
<td>Endothelial cells (mean ± SE)</td>
<td></td>
<td></td>
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<tr>
<td>MVD‡</td>
<td>15.8 ± 0.5</td>
<td>2.8 ± 0.2†</td>
</tr>
<tr>
<td>CD31/TUNEL (%)§</td>
<td>0</td>
<td>14.2 ± 1.1†</td>
</tr>
</tbody>
</table>

NOTE: ACC3 and ACCM cells were injected into the parotid glands of nude mice. Ten days later, mice were randomized and treatment started with vehicle or 50 mg/kg vandetanib. Mice were sacrificed after 12 d of treatment. Primary tumors were harvested and stained with specific antibodies.

* TUNEL positivity was quantified as the ratio of positively stained cells/total cells × 100 per field in five random 0.039-mm² fields at ×200 magnification.

† P < 0.01, compared with controls.

‡ MVD was determined by counting the number of completely stained blood vessels in 10 random 0.159-mm² fields at ×100 magnification.

§ CD31/TUNEL positivity was quantified as the ratio of CD31/TUNEL-positive cells/total endothelial cells in each of five random 0.039-mm² fields at ×200 magnification.
therapy. In contrast to squamous cell carcinoma of the head and neck, ACC frequently leads to distant metastasis. Hence, antiangiogenic therapy may be an attractive strategy for the treatment of ACC. Many of the angiogenesis inhibitors currently available have been designed to block the VEGF/VEGFR-2 signaling pathway, which is well known as a key regulator of tumor angiogenesis (30). Bevacizumab, a humanized anti-VEGF antibody, and small-molecule inhibitors of VEGFR-2 tyrosine kinase activity have been studied extensively in preclinical models and clinical trials (31). However, multiple signaling pathways are involved in tumor angiogenesis and progression. Therefore, single agents that can inhibit multiple targets, such as AEE788, vatalanib, and vandetanib, may have greater antitumor effects than agents targeting VEGF/VEGFR-2 signaling alone (12, 20, 32). By targeting two pathways (VEGFR-2 and EGFR), vandetanib offers the combined benefit of directly inhibiting tumor cell proliferation and survival like EGFR inhibitors and decreasing tumor angiogenesis by inhibiting VEGF signaling. Its good oral bioavailability supports once-daily oral therapy in vivo (33).

We showed that vandetanib (at concentrations <1 μmol/L) inhibited VEGFR-2 phosphorylation in the ACC3 cell line, which had high expression of VEGFR-2 in vitro. Although the functional significance of tumor-expressed VEGFRs is currently unclear, this finding suggests that these receptors may contribute to tumor progression by receiving growth and survival signals from the tumor cells themselves. In addition, we also showed that the treatment with vandetanib inhibited VEGFR-2 phosphorylation in the ACC tumors and their endothelial cells in vivo. Thus, anti-VEGF therapies may have both direct and indirect effects on tumor cells as well as effects on endothelial cells.

The primary antitumor effect of vandetanib is considered to be generated by its inhibition of VEGF signaling in tumor endothelial cells (20). However, our in vitro study showed that the agent directly inhibits tumor cell growth in ACC3, ACC2, and ACCM cells in a dose-dependent manner. The IC_{50} of vandetanib in these ACC cell lines is similar to those previously reported for various other types of tumor cells (2.7-13.5 μmol/L; ref. 20). This direct antiproliferative effect on tumor cells is thought to be due to inhibition of EGFR signaling because the IC_{50} of vandetanib in these assays is similar to that of EGFR tyrosine kinase inhibitors such as gefitinib (34). Vandetanib also has an antitumor effect by readily inducing apoptosis in the VEGF-2–expressing ACC3 cell line, whereas ACC2 and ACCM cells were less sensitive to induction of apoptosis by vandetanib. These differences in sensitivities between these cell lines may be attributed to different expression levels of EGFR tyrosine kinase; however, we believe that these relatively high IC_{50} values could also be due to nonspecific effects of the drug at these high concentrations.

Our in vivo findings that vandetanib reduced ACC3 tumor growth by 90% compared with control in the nude mice and that chronic administration of vandetanib was well tolerated are consistent with other reports of the agent in preclinical models of human lung, breast, prostate, ovarian, and colon cancers (20). We observed a similar inhibition of tumor growth in the mouse model with tumors grown from ACCM cells, which have a low level of VEGFR-2 and EGFR expression compared with ACC3 cells. These results are consistent with the concept that the antitumor effects of vandetanib in ACC may result primarily from inhibition of VEGF signaling and indirect antitumor effects rather than direct antiproliferative effects on the tumor.

Vandetanib may be used as part of a multimodality treatment scheme. Many preclinical studies suggest that vandetanib may provide synergistic effects when used in combination with radiation therapy, cytotoxic chemotherapy, and cyclooxygenase-2 inhibition (21, 35, 36). To extend these findings, we tested the in vivo effect of vandetanib + paclitaxel (Bristol-Myers Squibb). However, synergistic effects were not observed in these ACC models (data not shown). Given the poor response of ACC to conventional chemotherapy and radiation therapy, we
suggest that combined therapies that include vandetanib should be tested further to determine the maximal benefit that can be derived from these new antiangiogenic agents.

The antiangiogenic effect of vandetanib was evaluated by quantifying the tumor microvessels. We found that vandetanib significantly decreased the tumor microvessel number and size, which is consistent with the results of other studies done in lung, pancreatic, and gastric cancers (37–39).

To confirm whether the antitumor effects of vandetanib were due to its induction of tumor and tumor-associated endothelial cell death, we did a separate short-term study in which tumor tissues were taken from mice after 12 days of vandetanib treatment. Vandetanib induced significant apoptosis of tumor and endothelial cells, but the level of tumor-associated endothelial cell apoptosis was relatively low compared with that of tumor cell apoptosis.

Our findings of no difference in proliferating cell nuclear antigen staining between control mice and vandetanib-treated mice suggest that vandetanib-mediated inhibition of tumor cell proliferation is unlikely to be the primary mechanism of action of vandetanib in this model. Therefore, other mechanisms, such as antiangiogenesis and induction of apoptosis, likely are responsible for the observed vandetanib-mediated antitumor effects. Based on promising preclinical results, clinical trials are under way with vandetanib in a range of solid tumors, including lung, breast, and prostate (40, 41). A phase I study in Japanese patients showed that once-daily oral vandetanib at ≤300 mg/d was generally well tolerated (41). A phase I to II study of vandetanib plus docetaxel in platinum-pretreated patients with non–small cell lung cancer was recently completed with 50% of patients showing stable disease (42) and four phase III studies are currently ongoing in patients with advanced non–small cell lung cancer. Clinical trials with vandetanib are also under way in head and neck squamous cell carcinoma. In patients with previously untreated, unresected, stage III to IV head and neck squamous cell carcinoma, a phase I/II study of vandetanib in combination with radiation therapy and vandetanib in combination with radiation therapy and cisplatin chemotherapy is ongoing.7 However, no previous clinical trials have been conducted in patients with ACC.

In summary, vandetanib, an orally available agent that inhibits both EGFR and VEGFR tyrosine kinase activities, has shown significant antitumor activity in an orthotopic nude mouse model of ACC. Because ACC has shown little responsiveness to conventional chemotherapy and the long-term chances of recurrent and/or metastatic disease are relatively high, vandetanib warrants clinical assessment against this cancer.

Disclosure of Potential Conflicts of Interest

J.N. Myers has a commercial research grant from AstraZeneca.

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