Administration of VEGF receptor tyrosine kinase inhibitor increases VEGF production causing angiogenesis in human small-cell lung cancer xenografts

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Abstract. Angiogenesis is mediated mainly by vascular endothelial growth factor (VEGF), and VEGF causes rapid growth in cancers, including human small-cell lung cancer (SCLC). The anti-angiogenic strategy of treating cancer using VEGF receptor (VEGFR) inhibition is currently of great interest. We tested the effects of the VEGFR2 tyrosine kinase inhibitor (TKI) vandetanib on the proliferation of two kinds of SCLC cell lines: SBC-1 cells, with detectable VEGFR2 expression and MS-1-L cells, without detectable VEGFR2 expression. To evaluate the anti-tumor and anti-angiogenic effects of vandetanib *in vivo*, we grafted SBC-1 and MS-1-L cells into mice. After a 3-week treatment, we measured the tumor size and histologically evaluated necrosis and apoptosis using H&E and TUNEL staining, respectively. The microvessels in the xenografts were also quantified by immunostaining of CD31. Vandetanib did not affect the proliferation of SBC-1 cells, but stimulated the growth of MS-1-L cells. In the SCLC xenograft model, vandetanib inhibited growth and tumor angiogenesis in a dose-dependent manner in SBC-1 xenografts. Vandetanib inhibited the growth of MS-1-L xenografts at a low dose (<12.5 mg/kg/day), but it did not affect tumor size or change microvessel counts at a higher dose. Interestingly, secretion of VEGF increased significantly in the MS-1-L cell line in the presence of a high dose of vandetanib *in vitro*. The effects of vandetanib on tumor angiogenesis were different in SBC-1 and MS-1-L cell lines. Production of angiogenic factors such as VEGF by the tumor potentially stimulates tumor angiogenesis and results in the acquisition of resistance to VEGFR TKI.

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Introduction

Cancer therapy with high specificity and few adverse effects has been pursued since anti-cancer agents were introduced. Increasing our knowledge of cancer cell biology will help develop better cancer therapies. Low molecular weight compounds that inhibit specific functions of the malignant cells have been introduced to anti-cancer therapy. Some of these compounds were designed to block growth factor receptor signaling in human cancer by interacting with a specific portion of the growth factor receptors. Such compounds include small molecules that inhibit ligand-induced activation of the growth factor receptor tyrosine kinase, small molecule tyrosine kinase inhibitors (TKI) (1). These agents have the potential to develop into therapies with high specificity and low toxicity (2).

Several small molecule TKIs with anti-angiogenic properties are currently under preclinical as well as early clinical development. These TKIs include the vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitors vandetanib, sorafenib and sunitinib (3). Sorafenib and sunitinib have progressed beyond clinical trials in some tumor types and are licensed therapies in some markets. In a phase I study of patients with advanced solid tumors, daily oral vandetanib was generally well tolerated at daily oral doses of 300 mg/d or lower; notably, 4 of 9 Japanese patients with refractory non-small cell lung cancer (NSCLC) experienced objective tumor responses ranging from 90 to 438 days (4,5). Vandetanib in combination with docetaxel, increased progression free survival (PFS) compared with docetaxel alone in a phase II study of 127 patients with pre-treated advanced NSCLC. It was demonstrated that addition of vandetanib 100 mg improved median PFS from 12 weeks with docetaxel alone to 18.7 weeks in combination (HR=0.64, 95% CI 0.38-1.05; p=0.074). The combination therapy using vandetanib (100 mg) and docetaxel was generally well-tolerated; common adverse events included rash, diarrhea and asymptomatic QTc prolongation, all of which responded to a standard management (6). In that study, progression-free survival of patients receiving vandetanib treatment was significantly longer than that of those receiving gefitinib treatment.

SCLC is characterized by its rapid growth and tendency toward metastatic spread. SCLC cells have been reported to

express VEGF receptor 1 (VEGFR1), VEGFR2, and VEGFR3 (7). We previously reported that SCLC cell lines express large numbers of functional VEGFR2 receptors that play an important role in tumor growth and invasion (7). Previous studies have suggested that the VEGF/VEGFR system plays an important role in tumor growth and metastasis. VEGF has been reported to be a key growth factor in tumor angiogenesis (8-10). Therefore, inhibition of the VEGF/VEGFR system seems to be a promising strategy for cancer therapy. In fact, inhibition of the VEGF/VEGFR signaling pathway disturbs tumor angiogenesis and inhibits tumor growth by decreasing the vascular density (11,12). Both endothelial and parenchymal cells in tumors were killed mainly by induction of apoptosis (13-18). Therefore, VEGFRs in human SCLC may be a target for therapy using small molecules.

Vandetanib is a once-a-day oral anticancer agent that selectively inhibits VEGFR, epidermal growth factor receptor (EGFR), and RET (rearranged during transfection) signaling. Administration of vandetanib produced dose-dependent inhibition of tumor growth in nude mice bearing human NSCLC, prostate, breast, ovarian, colon and vulval tumors without SCLC (19-22). The IC_{50} of vandetanib against VEGFR2 tyrosine kinase activity was reported to be 40 nM, and vandetanib also inhibited VEGFR3 tyrosine kinase activity, which binds VEGF-C and VEGF-D (IC_{50} of 110 nM) (14). In contrast, the IC_{50} of vandetanib against VEGFR1 tyrosine kinase activity was 1600 nM. Vandetanib did not inhibit tyrosine kinase activity of other growth factor receptors, including platelet-derived growth factor receptor ß, FGFR1, c-erbB-2, c-kit, and type-1 insulin-like growth factor receptor (21). Ciardiello *et al* reported that vandetanib inhibited cell growth and induced apoptosis in human NSCLC, colon, ovarian, breast and gastric cancer cell lines that express both EGFR and TGF-α but lack VEGFR1 and VEGFR2. They suggested that vandetanib inhibited cell-proliferation in these cell lines by disturbing the transduction of the mitogenic signal from EGFR (23).

Among lung cancer patients treated with the EGFR TKI gefitinib, having the EGFR gene mutation, being a non-smoker, female, Asian, and having adenocarcinoma have been reported to be favorable factors in terms of good drug response (24-26). To intelligently adapt molecular targeting agents to cancer therapy, understanding the mechanism of anti-tumor activity is necessary. However, little is known about the effects of VEGFR2 TKIs on tumor angiogenesis. Therefore, in the present study we compared the effect of vandetanib on SCLC cell lines with and without VEGFR2 expression because VEGFR2 TKI inhibits tumor angiogenesis independent of the VEGFR2 expression levels of SCLC cells.

Materials and methods

Cell culture. SBC-1 and MS-1-L human SCLC lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). Cancer cells were cultured according to the supplier's recommendations. Briefly, cancer cells were grown in RPMI-1640 culture medium (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum. Cells were grown as suspension cultures at 37˚C in 95% air/5% CO_2 and passaged twice weekly in complete medium with a simple dilution of 1:5. Hypoxic culture conditions (1% O_2, 5% CO_2 and 94% N_2) were achieved by using a sealed hypoxia chamber (Billups-Rothenberg, Del Mar, CA, USA).

Reverse transcription (RT)-PCR. Total cellular RNA was extracted from the cell lines by using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) was transcribed into DNA using a reverse transcriptase. The PCR program used to amplify *VEGF*, *VEGFRs*, *EGFR* and *ß-actin* genes consisted of one preliminary cycle of 5 min at 94˚C, 45 sec at 60˚C, and 45 sec at 72˚C, followed by 30 cycles of 1 min at 94˚C, 45 sec at 65˚C, 2 min at 72˚C, and concluded with 7 min at 72˚C. The primers for *VEGF*, *VEGFRs*, *EGFR* and *ß-actin* gene amplification were: for *VEGF*, sense (5'-TCCAGGA GTACCCTGATGAG-3') and antisense (5'-CTTTCCTGG TGAGAGATCTGG-3') representing the immediate flanking region of the *VEGF* open reading frame involved in the alternative splicing of several exons; (27); for *VEGFR1*, sense (5'-ATTTGTGATTTTGGCCTTGC-3') and antisense (5'-CA GGCTCATGAACTTGAAAGC-3') (28); for *VEGFR2*, sense (5'-GTGACCAACATGGAGTCGTG-3') and antisense (5'-CCAGACATTCCATGCCACTT-3') (28); for *VEGFR3*, sense (5'-CCCACGCAGACATCAAGACG-3') and antisense (5'-TGCAGAACTCCACGATCACC-3') (29); for *EGFR*, sense (5'-TGACTCCGTCCAGTATTGATCG-3') and antisense (5'-ATTCCTTACACACTTTGCGCC-3') (30); and for *ß-actin*, sense (5'-GGAAATCGTGCGTGACATT-3') and antisense (5'-CATCTGCTGGAAGGTGGACAG-3') (30). RT-PCR was done using a one-step RT-PCR kit (Qiagen). The bands were visualized by ethidium bromide staining.

Xenograft tumor model. SCLC cell lines, SBC-1 and MS-1-L, were inoculated into the backs of 5-week-old female athymic BALB/c-nu/nu mice (Charles River Laboratories Japan, Inc., Yokohama, Japan). The mice were maintained for about 1 week until xenografts reached 2 mm x 2 mm x 2 mm. Then the mice were randomly divided into 4 groups and treated with vandetanib 12.5 mg/kg/day, 25 mg/kg/day, 50 mg/kg/day, or the vehicle only. Each group included 4 mice. Tumor diameter and body weight were measured twice weekly. We measured tumor size with calipers, and the volume was calculated as: length x width^2 x 0.5. Xenografts that were smaller than pretreatment size were defined as tumor reduction. Three weeks after the beginning of the treatment, the xenografts were removed, and the specimens were processed for pathological analyses.

Pathological analyses. For hematoxylin-eosin (H&E) staining and deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining, the xenografts were fixed in 10% neutral buffered formalin and embedded in paraffin. We performed TUNEL staining using the DeadEnd Colorimetric TUNEL system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The sections were then observed, and representative images were taken

under a microscope (Olympus, Tokyo, Japan). A cell with a brown-stained nucleus was recorded as TUNEL positive.

Microvessel counts assay. CD31 immunostaining of tumors was done using 10 μm frozen sections at -80˚C to determine microvessel counts (MVC). Briefly, the frozen sections were incubated overnight with a 1:50 dilution of monoclonal rat anti-mouse CD31 (PECAM-1) antibody (BD PharMingen, San Diego, CA, USA) at 4˚C. After washing with phosphate-buffered solution (PBS), a 1:500 dilution of FITC-labeled rabbit anti-rat antibody was added. Microvessels stained with CD31 were counted in five different areas of the xenografts without necrosis at high magnification (x200), and then the five values were averaged for each sample as the MVC.

Cell proliferation assay. Cell proliferation was determined by MTS assays and cell counts. A CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega) was used to evaluate the cytotoxicity. Samples (100 μl) containing $2.5x10^4$ exponentially growing cells were added to each well of a 96-well plate. PBS for the control and 0.001 μM to 100 μM concentrations of vandetanib were added to the wells. After incubation for 72 h at 37˚C, 20 μl of CellTiter 96 AQueous One Solution was added to each well, and the plates were further incubated for 4 h at 37˚C. Absorbance was measured at 490 nm with a 96-well plate reader. Each experiment was performed in eight replicate wells for each drug concentration. Experiments were repeated three times.

Assay of culture medium protein. Briefly, $2.5x10^6$ cells were plated in 10-cm dishes using complete medium, and vandetanib or only the vehicle was added. Cells were incubated at 37˚C for 72 h. At the end of the incubation, the culture supernatant was removed and stored at -80˚C. Protein levels were analyzed using a commercial antibody array (Raybio Growth Factor Antibody Array I; AAH-GF-1-4, RayBiotech Inc., Norcross, GA, USA) that allows for quantitative determination of the protein levels of a variety of growth and proangiogenic factors. Hybridization and detection were performed following the manufacturer's instructions using 1 ml of culture medium. Digital chemiluminescence images were taken by a Luminescent Image Analyzer LAS-3000 (Fuji Film Inc., Tokyo, Japan). Quantification of the spot intensities was performed using Multi-Gauge software (Fuji Film Inc.).

The levels of VEGF in the cell culture supernatants were determined using standard sandwich ELISA methods (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The VEGF concentration in the media was determined from the standard curve; the detection limit was 0.64 pg/ml. Results were obtained from three independent experiments.

DNA extraction and sequencing. DNA was extracted from SCLC cell lines using a DNasey Mini Kit (Qiagen). Gene regions for the TKI binding sites of *EGFR* and *VEGFR2* genes were amplified using the forward and reverse primers previously reported (25). Cycle sequencing was done using a Big-Dye sequencing kit (Perkin-Elmer Life And Analytical Sciences Inc., Wellesley, MA, USA), then the sequences were analyzed using an ABI 310 Genetic Analyzer (Perkin-Elmer). The DNA sequences were compared with the genomic sequence data in GenBank databases.

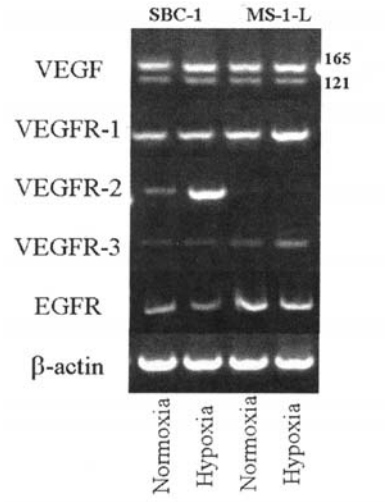


Figure 1. Expression of VEGF isoforms, VEGF-Rs and EGFR, by small-cell lung cancer cell lines determined by reverse transcription-PCR.

Reagents. Vandetanib (Zactima™; ZD6474) was provided by AstraZeneca Pharmaceuticals Ltd. (Macclesfield, UK).

Image analysis. Areas with necrosis were measured using computer planimetry (Scion Image, Scion Corp., USA), then expressed as percentage of the total area of the xenograft.

Statistical analyses. Differences in tumor volume and MVC values among each experiment group were analyzed by the χ^2 test. The difference was considered to be statistically significant when the p-value was <0.05. Statview 5.0J software (Abacus Concepts, Inc., Berkeley, CA, USA) was used for all analyses.

Results

Expression of VEGF, VEGFR and EGFR in SCLC cell lines. The predominant isoform of VEGF in SBC-1 and MS-1-L cells was determined by RT-PCR. It was previously reported that transcriptions of hypoxia-related genes like VEGF were induced by hypoxic conditions mediated by HIF-1α (31), and a slight up-regulation of VEGF mRNA by hypoxia was observed in both cell lines. SBC-1 cells expressed *VEGFR2* mRNA, whereas MS-1-L cells did not (Fig. 1). The level of *VEGFR2* mRNA in SBC-1 cells was enhanced under hypoxic culture conditions. However, *VEGFR2* mRNA was not detectable in MS-1-L cells even under hypoxic culture conditions. Both SBC-1 and MS-1-L cells expressed *VEGFR1* and *VEGFR3* mRNAs. *EGFR* mRNA was also detectable in SBC-1 and MS-1-L cell lines under both normoxic and hypoxic culture conditions (Fig. 1).

Effects of vandetanib on SCLC xenografts. Oral administration of vandetanib decreased the tumor volume of

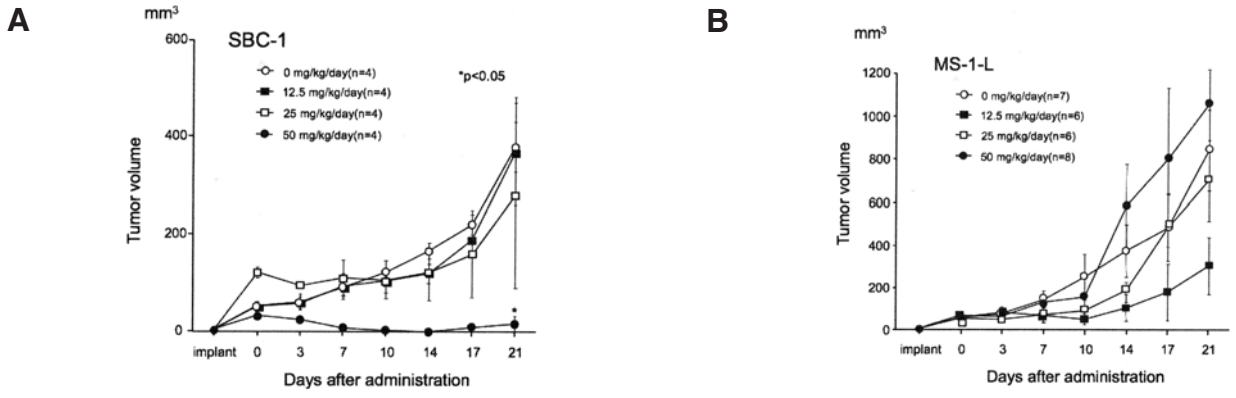


Figure 2. Effects of vandetanib on growth of small-cell lung cancer xenografts in athymic mice. (A) SBC-1 xenografts; (B) MS-1-L xenografts. n, 4 each.

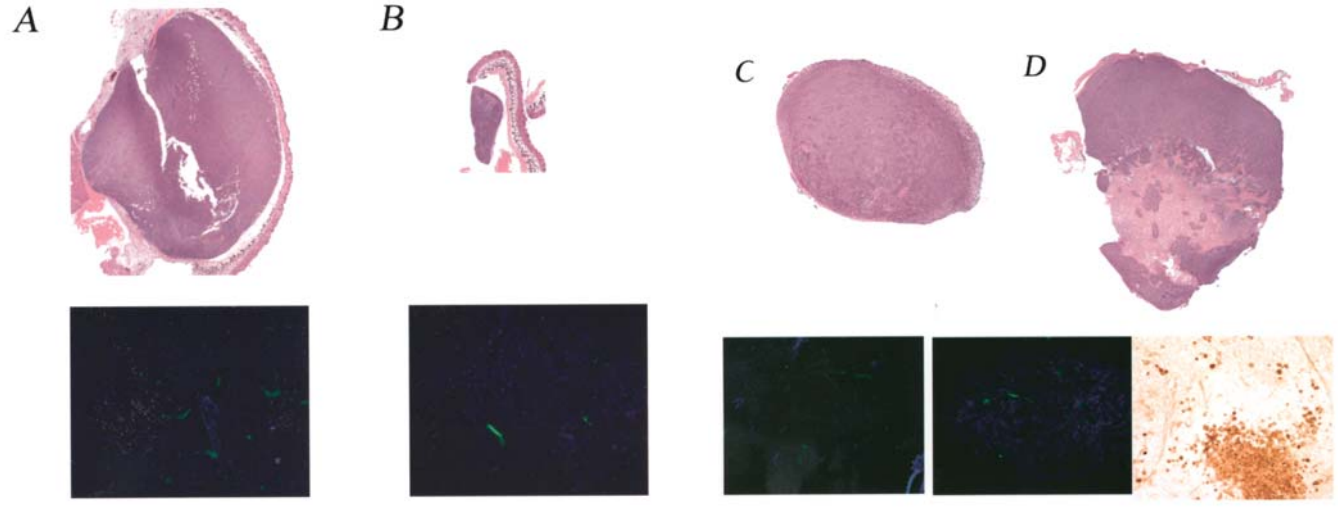


Figure 3. Pathology of xenografts. (A and B) H&E staining of SBC-1. (C and D) H&E staining of MS-1-L xenografts (x40). Microvessels determined by CD31 immunostaining are shown in the lower panels of each figure (x200). Apoptotic staining is shown in the bottom right panel of D. (A and C) vehicle only; (B and D) 50 mg/kg/day vandetanib.

SBC-1 in a dose-dependent manner (Fig. 2A). At a dose of 50 mg/ kg/day, vandetanib completely inhibited tumor growth. In MS-1-L tumor-bearing mice, the tumor volume was attenuated at 12.5 mg/kg/day of vandetanib; however, at a higher dose (>25 mg/kg/day) of vandetanib, the tumor volume was increased (Fig. 2B).

Neither liver metastasis nor lung metastasis was found in any animal. No apparent side effect (e.g., loss of body weight, reduced diet consumption) was observed at a dose up to 50 mg/kg/day.

Pathological findings of SCLC xenografts. In SBC-1 mouse xenografts treated with only the vehicle, H&E staining showed small degenerative and necrotic areas in the tumors (Fig. 3A). In the group administered 50 mg/kg/day, tumors were decreased in size and showed only small degenerative and necrotic changes (Fig. 3B).

In MS-1-L mice, xenografts stained with H&E showed hypercellularity with nuclei crowding but smaller degenerative and necrotic areas in mice treated with only the vehicle (Fig. 3C). In the 12.5 mg/kg/day administration group, a reduction in tumor volume without internal degeneration was seen. In groups administered 25 mg/kg/day and 50 mg/kg/day, differentiation of the areas of necrosis within the tumor was simple because of its clear margin (Fig. 3D). Bottom panels of each figures show representative examples of microvessels immunostained with CD31 (green) and nuclei (blue) in xenografts treated with 50 mg/kg/day vandetanib or its vehicle. Abundant microvessels were observed in vehicle controls of SBC-1 and MS-1-L xenografts (Fig. 3A and C, bottom); however, the group administered vandetanib at 50 mg/kg/day of SBC-1 showed fewer microvessels, while those of the MS-1-L xenografts were not affected. The relative necrotic areas of the tumor tissue in the MS-1-L xenografts were 20.34±5.24% (average ± SD) in 50 mg/kg/day group and 4.25±1.08% in group with only vehicle. Degeneration and necrosis with hemorrhagic foci were seen in 20.3% of the tumor areas of mice that received 50 mg/kg/day vandetanib. The apoptotic index measured in the viable area of the tumors using TUNEL staining showed a significant induction of apoptosis in mice that received 50 mg/kg/day vandetanib (Fig. 3D bottom right).

Tumor angiogenesis in SCLC xenografts. Tumor angiogenesis was estimated by assessment of the number of MVC in several sequential slides. Vandetanib caused dose-dependent

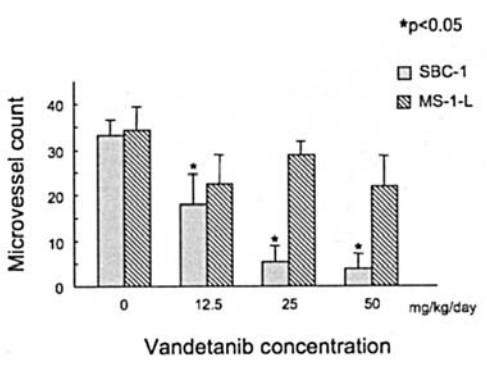


Figure 4. Results of microvessel count.

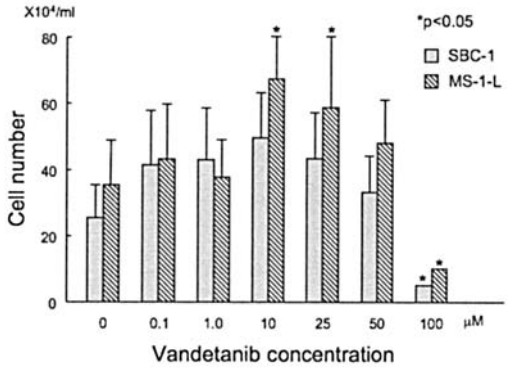
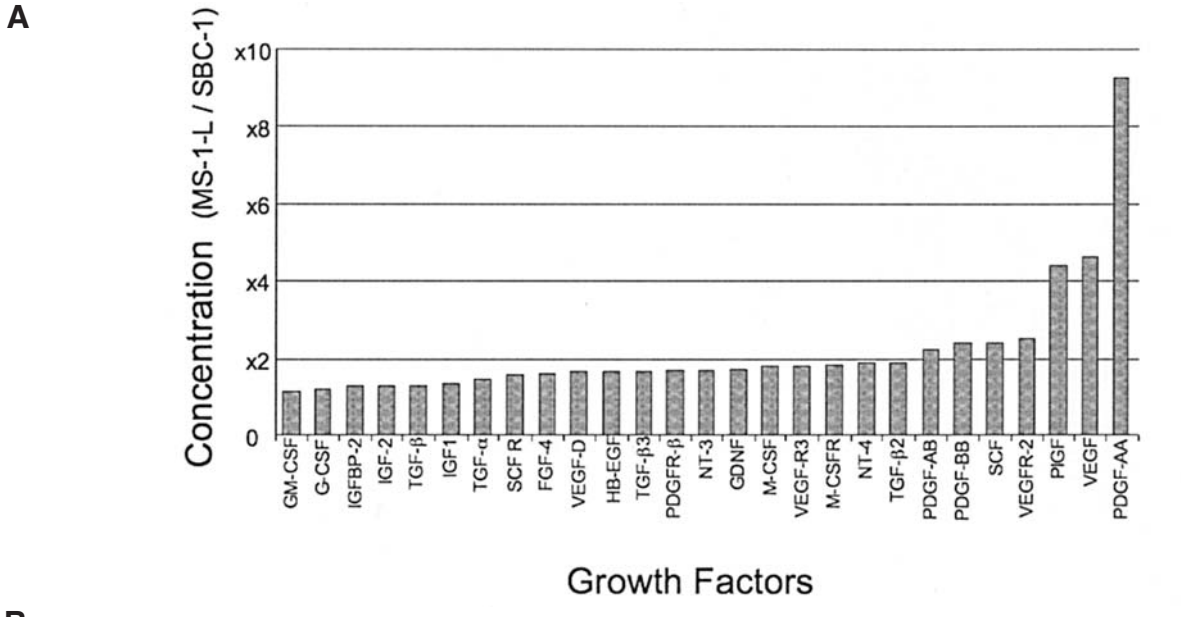


Figure 5. Effects of vandetanib on proliferation of human small-cell lung cancer cell lines.



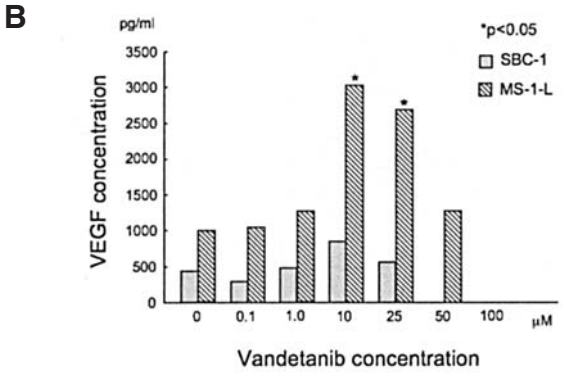


Figure 6. (A) Production of proangiogenic growth factors measured by antibody array. (B) VEGF concentrations in culture medium measured by ELISA.

inhibition of tumor angiogenesis in the SBC-1 xenografts. In contrast, vandetanib administration did not significantly change the number of MVC in the viable areas of MS-1-L xenografts (Fig. 4).

Cell growth inhibition by vandetanib. The effects of vandetanib on cellular proliferation of these cells were determined by a cell count assay. Cellular growth in SBC-1 cells was not affected by vandetanib (up to 10 μM). In contrast, vandetanib stimulated cell growth at the high dose (100 μM) and attenuated cell proliferation, even inducing cell damage (apoptosis) in both cell lines (Fig. 5). Cell proliferation was also assessed by MTS assay, which showed similar results.

Production of proangiogenic factors in cells. To determine the difference in production of growth factors and proangiogenic factors between SBC-1 and MS-1-L, we analyzed the growth medium of these cell lines after a 2-day incubation with vandetanib using an antibody array that quantifies the levels of 41 growth factors (Fig. 6A). The data showed that vandetanib-induced production of PDGFs (-AA, -AB, -BB), VEGF, PlGF, soluble VEGFR2, and SCF proteins by MS-1-L was more than double those by SBC-1 in the presence of vandetanib. These results suggest that up-regulation of other proangiogenic factors compensates for the blocking of VEGFR downstream signaling pathways.

The concentration of VEGF in the medium was measured by ELISA. The VEGF level was increased after the 2-day incubation without vandetanib. The addition of vandetanib

increased the VEGF level in the medium of MS-1-L cells, but it did not increase the VEGF level in that of SBC-1 cells (Fig. 6B).

DNA sequencing of EGFR and VEGFR2 genes. In both cell lines, DNA sequences around the ATP-binding pockets of the EGFR and VEGFR genes were similar to those in the genomic sequence data in GenBank databases (data not shown).

Discussion

In the present study, vandetanib did not significantly inhibit cell proliferation in human SCLC cell lines with detectable VEGFR2 expression. Tanno *et al* (7) showed that cultured human SCLC cell lines express functioning VEGFR2. In this study, treatment with anti-human VEGFR2 antibody did not significantly inhibit cell proliferation of human SCLC cells with VEGFR2 expression, but it inhibited migration of these cancer cells. Therefore, VEGFR2 seemed to have relatively little significance in SCLC cell proliferation. In contrast, vandetanib slightly stimulated growth of MS-1-L cells, which did not have detectable VEGFR2 expression. These results suggest the possibility that the effects of VEGFR2 inhibitors differ between human SCLC cells with high VEGFR2 expression and those with low VEGFR2 expression.

We found that vandetanib significantly inhibited the growth of inoculated SBC-1 cells, which had detectable VEGFR2 expression, in a dose-dependent manner. However, vandetanib enlarged MS-1-L tumors *in vivo*. There was no evidence of a statistically significant difference between any of the treatment groups in either of the xenograft studies. These results suggested that SBC-1 cells were sensitive and MS-1-L cells aquired *de novo* resistance to vandetanib when they were inoculated into nude mice. Paez *et al* reported that the effect of an EGFR inhibitor, gefitinib, depends on mutation of the *EGFR* gene within the ATP-binding region (25). It was reported that vandetanib directly binds to the ATP-binding sites of VEGFR and EGFR, and its effect is dependent upon the mutation in the *EGFR* gene (32).

Therefore, the sensitivity of cancer cells to tyrosine kinase inhibitors is dependent on the mutation status around the inhibitor binding site. In order to examine whether the different effects of vandetanib on SBC-1 and MS-1-L cells are caused by a mutation in *EGFR*, we sequenced the ATP-binding region of both *EGFR* and *VEGFR2* genes. There were no gene mutations around the ATP-binding site of the *EGFR* and *VEGFR2* genes of either cell line. Thus, gene mutation around the ATP-binding site of these genes did not explain the resistance of MS-1-L cell line to vandetanib.

Inhibition of tumor angiogenesis seemed to be the main reason for the growth inhibition by vandetanib in SBC-1 xenografts because the compound did not significantly inhibit SBC-1 cell growth *in vitro*. However, it decreased the MVC in the present study. In contrast, both tumor size and necrotic area were significantly increased in MS-1-L xenografts by vandetanib administration. These data suggest that the effects of vandetanib on tumor angiogenesis were different in SBC-1 and MS-1-L cell lines.

Molecular mechanisms of resistance to molecular targeting agents have been reported and include increased angiogenesis, activation of alternative tyrosine kinase receptor pathways, and the constitutive activation of downstream mediators (33). Engelman *et al* reported that cells resistant to EGFR kinase inhibitors displayed amplification of the MET oncogene and maintained activation of ERBB3/PI3K/Akt signaling in the presence of gefitinib. They suggested the possibility that MET amplification promotes drug resistance in other ERBB-driven cancers (34). Ciardiello *et al* reported that chronic administration of EGFR mAbs or TKIs to athymic mice bearing human GEO colon cancer caused development of resistant cancer cells. In their study, resistant colon cancer cells showed 5-10-fold increase in VEGF expression, as detected by the Western blotting. They suggested that the up-regulated VEGF expression contributed to the increased angiogenesis and resulted in the development of resistance to the EGFR inhibitors (35). In clinical studies of cetuximab against human colon cancer, an increase in EGFR mRNA as well as VEGF mRNA was reported to be correlated with acquisition of resistance to cetuximab (35,36). Therefore, we compared the effects of vandetanib on production of proangiogenic growth factors including VEGF and tumor angiogenesis between SBC-1 and MS-1-L xenografts.

Our antibody array data showed that vandetanib induced more PDGF, VEGF, PlGF, VEGFR2, and SCF peptide production in MS-1-L than in SBC-1 cells in the presence of vandetanib. However, the levels of FGF family peptides did not increase in either cell line. These results supported observations by Casanovas *et al* (37). They reported that the acquisition of resistance to VEGF receptor antibody correlated with an increase in tumor angiogenesis in a mouse pancreatic islet carcinogenesis model. Acquisition of the resistance was independent of the VEGF system; however, involvement of other proangiogenic systems, including the FGF system, was suggested. When both FGF and VEGF signal transduction were blocked simultaneously, tumor progression was no longer observed (37).

Interestingly, secretion of VEGF was significantly increased in the MS-1-L cell line by vandetanib administration in the present study. This increased VEGF production by MS-1-L tumor cells probably stimulated tumor angiogenesis and resulted in rapid enlargement of the tumor. Although the MVC was not significantly increased, the necrotic area was significantly increased in MS-1-L xenografts. Noguera-Troise *et al* reported the effects of an anti-angiogenic factor, Delta-like ligand 4 antibodies using a mouse xenograft model. In their study, the MVC did not correlate with tumor size and hypoxic area (38). Our results support their observations.

Recruitment of vascular endothelial cells by VEGF secretion has been reported to be one of the most important processes of tumor angiogenesis (39-41). VEGFR inhibitors including vandetanib inhibit this recruitment of vascular endothelial cells, thus disturbing the angiogenesis within malignant tumors (42). Tumor angiogenesis consists of angiogenesis and vasculogenesis (39). Angiogenesis involves the recruitment of sprouting vessels from existing vessels, and vasculogenesis involves the incorporation of endothelial progenitors into the growing vascular bed. Although a number of angio-

genic factors have been identified, VEGF has been recognized as a key factor in both angiogenesis and vasculogenesis. VEGF mobilizes endothelial progenitor cells (EPCs) from the bone marrow. These EPCs are reported to play an important role in neovascularization. Asahara *et al* reported that EPCs positive for CD34 as well as VEGFR2 were isolated from the region with neovascularization (39). Rafii *et al* reported that treatment with an anti-VEGFR1 antibody reduced recruitment of EPCs in the tumor angiogenesis using a tumor-bearing mouse model (41). These results suggest that multiple angiogenic factors and receptors play roles in tumor angiogenesis depending on the environment of the neovascularization.

In the present study, MS-1-L was resistant to the VEGFR2 TKI vandetanib. We speculated that increased production of VEGF by the human SCLC xenograft probably induced tumor angiogenesis (controlled by VEGFR1 in MS-1-L) in the presence of vandetanib because the IC_{50} of vandetanib against VEGFR1 is higher than that against VEGFR2 (21). It is also possible that tumor vasculogenesis was blocked by the inhibitor; however, early stage angiogenesis proceeds via the activity of other angiogenic factors besides the VEGF family. This could be supported by the present results that the area of necrosis was increased in MS-1-L by vandetanib administration. The lack of coordination in the tumor angiogenesis possibly caused incomplete vasculogenesis in MS-1-L xenografts, causing hypoxia in the inner portion of the MS-1-L xenografts.

We observed slight stimulation of MS-1-L cell growth by vandetanib *in vitro*. Increased production of VEGF from MS-1-L cells may explain this result. However, because VEGF did not stimulate SCLC cell proliferation in the previous study, production of other growth factors from MS-1-L cells due to a block of VEGFR2 signal transduction remains a possible explanation for the growth stimulation of MS-1-L cells in the present study. Extensive studies of peptide production from cancer cells in the presence of molecular targeting agents will be of interest.

Our present results raise the possibility that treatment using VEGFR2 TKI stimulates tumor growth, depending on the VEGFR2 expression status of the tumor. Production of angiogenic factors such as VEGF by the tumor potentially stimulates angiogenesis. According to our present results, addition of growth factor inhibitors such as the anti-VEGF antibody bevacizumab may overcome resistance to therapy using a growth factor receptor inhibitor such as vandetanib.

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