Combining the Multitargeted Tyrosine Kinase Inhibitor Vandetanib with the Antiestrogen Fulvestrant Enhances Its Antitumor Effect in Non-small Cell Lung Cancer

Jill M. Siegfried, PhD, Christopher T. Gubish, MS, Mary E. Rothstein, BS, Cassandra Henry, BS, and Laura P. Stabile, PhD

Introduction: Estrogen is known to promote proliferation and to activate the epidermal growth factor receptor (EGFR) in non-small cell lung cancer (NSCLC). Vascular endothelial growth factor (VEGF) is a known estrogen responsive gene in breast cancer. We sought to determine whether the VEGF pathway is also regulated by estrogen in lung cancer cells, and whether combining an inhibitor of the ER pathway with a dual vascular endothelial growth factor receptor (VEGFR)/EGFR inhibitor would show enhanced antitumor effects.

Methods: We examined activation of EGFR and expression of VEGF in response to β-estradiol, and the antitumor activity of the multitargeted VEGFR/EGFR/RET inhibitor, vandetanib, when combined with the antiestrogen fulvestrant both in vitro and in vivo.

Results: NSCLC cells expressed VEGFR-3 and EGFR. Vandetanib treatment of NSCLC cells resulted in inhibition of EGFR and VEGFR-3 and inhibition of β-estradiol-induced P-MAPK activation, demonstrating that vandetanib blocks β-estradiol-induced EGFR signaling. Treatment with β-estradiol stimulated VEGFA mRNA and protein ($p < 0.0001$ over baseline), suggesting estrogenic signaling causes heightened VEGFA pathway activation. This estrogenic induction of VEGFA mRNA seems largely dependent on cross-talk with EGFR. Long-term vandetanib treatment also significantly increased ERβ protein expression. The combination of vandetanib with fulvestrant maximally inhibited cell growth compared with single agents ($p < 0.0001$) and decreased tumor xenograft volume by 64%, compared with 51% for vandetanib ($p < 0.05$) and 23% for fulvestrant ($p < 0.005$). Antitumor effects of combination therapy were accompanied by a significant increase in apoptotic cells compared with single agents.

Conclusions: Fulvestrant may enhance effects of vandetanib in NSCLC by blocking estrogen-driven activation of the EGFR pathway.

Key Words: EGFR, Estrogen, NSCLC, VEGF, VEGFR, Tyrosine kinase inhibitor.

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Department of Pharmacology & Chemical Biology, University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, Pennsylvania.
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Address for correspondence: Laura P. Stabile, Hillman Cancer Center, UPCI Cancer Pavilion, Office 441, 5117 Centre Avenue, Pittsburgh, PA 15213-1863. E-mail: las22@pitt.edu

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Non-small cell lung cancer (NSCLC) is the leading cause of cancer deaths in the United States and worldwide, with a 15% 5-year survival rate for all stages combined.[1] Currently, the best available first-line chemotherapy treatment regimens for metastatic NSCLC achieve only a median 8- to 12-month survival time.[2,3] Targeted therapies, such as those inhibiting the epidermal growth factor receptor (EGFR), have been introduced for second-line treatment of NSCLC. Erlotinib is a tyrosine kinase inhibitor (TKI) targeting the EGFR, a receptor frequently expressed in NSCLC. Erlotinib has demonstrated a high response rate and increased survival in certain lung cancer patient populations such as never smokers and those with EGFR mutations, and evidence of activity in some patients with wild-type EGFR.[4] Molecularly targeted agents such as erlotinib show less toxicity compared with chemotherapy. These drugs can improve survival in sensitive patients, but resistance often develops, limiting clinical utility.[4] Small molecule TKIs with a broader spectrum of targets may be useful against tumors that show high activity of more than one kinase known to promote oncogenesis.

Tumor angiogenesis is the process leading to the formation of blood vessels within a malignancy through stimulation of endothelial cells. Angiogenesis plays a key role in cancer cell survival, localized tumor growth, and metastases.[5] Vascular endothelial growth factors (VEGFs) are potent mitogens for endothelial cells that activate the angiogenic switch in vivo and enhance vascular permeability. Up-regulation of VEGFs causes increased neovascularization in many tumor types.[6] There are several VEGF family members known. VEGFA, the major form responsible for angiogenesis, binds two distinct receptors in vascular cells, vascular endothelial growth factor receptor (VEGFR)-1 and VEGFR-2, whereas VEGFB only recognizes VEGFR-1.[7] VEGFR-2 is considered to be the dominant signaling receptor for endothelial cell proliferation, permeability, and differentiation in blood vessels.[8] VEGFR-3 (also known as Flt-4) is needed for angiogenesis that occurs in lymphatic endothelial cells,[9] and primarily recognizes the ligands VEGFC and VEGFD. VEGFR-3 expression has also been detected in gastric tumor cells[10] and in lung tumor cells,[11,12] where it seems to be expressed along with VEGF ligands. Malignant

cell proliferation and survival may be regulated in these tumors by VEGFC or D in an autocrine manner.

Targeting VEGFR-3 in addition to VEGFR-2 may inhibit actions of VEGF ligands on both endothelial and tumor cells. Vandetanib is an orally active small molecule anilinoquinazoline derivative that is a potent inhibitor of the VEGFR-2 tyrosine kinase (IC50 40 nM) with additional activity against EGFR (IC50 500 nM), VEGFR-3 (IC50 110 nM), and RET (IC50 130 nM).[13] Although EGFR and VEGFR pathways are commonly involved in NSCLC, the RET gene does not seem to play a large role in lung cancer. Vandetanib has shown activity against medullary thyroid cancer, a disease in which the RET gene is frequently mutated.[14] Despite promising phase II results in lung cancer, recently completed randomized phase III trials of vandetanib in combination with pemetrexed (ZEAL) or docetaxel (ZODIAC) or as a monotherapy versus erlotinib (ZEST) in advanced lung cancer did not show increased overall survival compared with any of these agents alone.[15] Nevertheless, there may be other settings in which vandetanib could be effective against lung cancer when combined with other targeted agents.

We have previously elucidated the role of estrogen receptors (ERs) in NSCLC[16] and have demonstrated cross-talk between the ER and EGFR pathways in lung cancer.[17] The primary means of cross-talk we detected in NSCLC was due to release of EGFR ligands by estrogen which activated the EGFR pathway.[17] Others have reported an ER-EGFR cross-talk in which ERβ can be phosphorylated by EGFR or other kinases that converts it to an active signaling molecule causing induction of estrogen responsive genes.[18] EGFR protein expression was also down-regulated in response to β-estradiol and up-regulated in response to fulvestrant. Furthermore, the combination of the EGFR TKI gefitinib with the antiestrogen fulvestrant reduced NSCLC proliferation and increased apoptosis in vitro and in vivo, compared with either agent alone. Furthermore, we have previously demonstrated that ERβ is the main functional ER isoform involved in lung cancer proliferation and signaling whereas ERα is rarely expressed.[19] We recently completed a pilot phase I clinical trial, which established the safety of combining gefitinib and fulvestrant in postmenopausal women with advanced NSCLC.[20] The combination therapy proved safe and well tolerated with a median progression-free survival of 12 weeks and a median overall survival of 33 weeks, although gefitinib has since been withdrawn as a treatment choice for lung cancer in the United States. In a preliminary analysis, ERβ positivity in at least 60% of tumor cells correlated with a higher overall survival in patients treated with the combination. ERβ is expressed by $>$90% of both male and female NSCLC,[21] and EGFR-ERβ cross-talk may be an important aspect of NSCLC growth. Because VEGFs are estrogen-responsive genes in breast cancer,[22] VEGFs may also be regulated in part by the estrogen pathway in NSCLC. This could be due to induction of VEGF gene expression by activation of ERβ, activation of EGFR through ERβ cross-talk, or both.

To address the potential of inhibiting multiple interdependent growth-stimulatory pathways simultaneously in NSCLC, here, we examine interaction between the main form of estrogen, β-estradiol, and VEGFs, and the antitumor activity of the multitargeted VEGFR/EGFR inhibitor, vandetanib, when combined with the antiestrogen fulvestrant. Treatment with β-estradiol increased secretion of VEGFA in NSCLC cells, which was largely mediated by ER activation of the EGFR. Vandetanib could block β-estradiol-induced EGFR activation, and blockade with vandetanib led to up-regulation of ERβ expression. The combination of fulvestrant and vandetanib showed significantly increased antitumor activity in NSCLC cells containing either wild-type EGFR or a rare EGFR mutation not associated with enhanced sensitivity to EGFR TKIs. Tumor growth in xenografts of NSCLC containing wild-type EGFR was maximally inhibited by a combination of vandetanib and fulvestrant. Malignant cells within lung tumor xenografts were found to express the receptors EGFR, VEGFR-3, and ERβ, whereas the receptor VEGFR-2 was expressed by tumor vasculature. The ligands β-estradiol and VEGFA were also detected in xenografts. These observations suggest that the interacting pathways targeted by vandetanib and fulvestrant are present in proliferating lung tumors. Using an antiestrogen to block both estrogen-dependent stimulation of VEGFA expression and estrogen-dependent activation of EGFR, and a possible up-regulation of ERβ in response to EGFR inhibition, may enhance the anticancer activity of vandetanib in lung cancer.

MATERIALS AND METHODS

Cell Lines and Reagents

201T and 273T cells were established as described previously.[23] A549, K562, and TT cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). 201T and A549 cells are wild-type for EGFR and EGFR TKI resistant, whereas 273T cells contain a rare point mutation of EGFR (Y727C), which shows minimal increased sensitivity to EGFR TKIs.[24] Human umbilical vein endothelial cells (HUVEC) and A431 cell lysates were purchased from Santa Cruz Biotechnology. Gefitinib was purchased from ChemiTek (Indianapolis, IN). Fulvestrant was purchased from Tocris (Ellisville, MO). Vandetanib was provided by AstraZeneca under a material transfer agreement. β-estradiol was from Sigma-Aldrich (St. Louis, MO). EGFR blocking antibody, C225 was obtained from Imclone Systems, Inc. (New York, NY). VEGF enzyme-linked immunosorbent assay (ELISA) kits and recombinant human EGF were purchased from R&D Systems (Minneapolis, MN). 201T, 273T, and A549 cells were authenticated by short tandem repeat (STR) profiling using the AmpFlSTR Identifiler PCR amplification kit (Applied Biosystems) within 6 months of performing the experiments. K562 and TT cells were used in experiments after purchase from ATCC within 6 months. ATCC authenticated K562 and TT cells by STR profiling. TT cells were grown in F-12K medium plus 10% fetal bovine serum (FBS), A549 cells were grown in BME +1% FBS, K562 cells were grown in Iscove's modified Dulbecco's medium +10% FBS. 201T and 273T cells were grown in BME +10% FBS.

Protein Extraction and Western Analysis

Lung cancer cells or frozen tumor xenografts were lysed in ice cold radioimmunoprecipitation buffer (10 mM Tris-HCl [pH, 7.6], 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 1 mM sodium vanadate, and protease minitab) and sonicated. Insoluble material was removed by centrifugation (14,000 rpm) for 10 minutes at 4°C. Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose and probed overnight at 4°C with primary antibody. Antibodies used include the following: rabbit anti-VEGFR-2 (1:1000, 55B11; Cell Signaling Technology, Beverly, MA), mouse monoclonal anti-Flt-4 (VEGFR-3) (1:200, D-6, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-EGFR (1:500, 1005; Santa Cruz Biotechnology), rabbit anti-RET (1:1000, 3220, Cell Signaling Technology), mouse antiactin (1:10,000, Millipore, Billerica, MA), rabbit anti-P-MAPK (1:1000, 9101; Cell Signaling Technology), and rabbit anti-P-AKT (1:1000; 9271; Cell Signaling Technology). Immunoreactivity was detected using antimouse or antirabbit IgG conjugated peroxidase and visualized by chemiluminescence.

For induction of P-MAPK and P-Akt, lung cancer cells were grown to 75% confluency and serum deprived for 48 hours. Cells were treated as described in the figure legends. Protein extracts were prepared and analyzed as described above. Primary antibodies were anti-P-MAPK (1:1000, 9101; Cell Signaling Technology), anti-total MAPK (T-MAPK) (1:1000, 9102; Cell Signaling Technology), anti-P-AKT (1:1000; 9271; Cell Signaling Technology), and anti-actin (1:10,000; Millipore). Quantitation was done by densitometry and ImageQuaNT analysis and expressed as the ratio of P-MAPK to T-MAPK or P-Akt to β-actin with control set to 100 or 1.

For detection of induction of ERβ protein, cells were grown to 75% confluency in phenol-red free medium. Cells were treated with vandetanib (2.5 μM) or DMSO control in phenol-red free medium with 1% charcoal stripped serum and replenished daily for 7 days. Protein extracts were prepared on days 1, 4, and 7 as described above. Primary antibody was rabbit anti-ERβ (1:1000, clone 68−4, Millipore). The blot was stripped and reprobed with actin antibody. Quantitation was done by densitometry and ImageQuaNT analysis and expressed as the ratio of ERβ to actin. No treatment on day 1 was set to 1.

VEGF ELISAs

Cells were treated as indicated in the figures. Cell culture supernatant was removed and equal amounts were analyzed in duplicate following the manufacturers' instructions using a human VEGFA, VEGFC, or VEGFD Quantikine ELISA kit that detects the specific VEGF isoform, with no reactivity against other VEGF isoforms. Total protein was used to normalize all ELISA data.

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction

Cells were serum starved for 48 hours and then treated with 10 nM β-estradiol from 0 to 24 hours. In Figure 3*C*, cells were pretreated with inhibitors as indicated followed by treatment with 10 nM β-estradiol for 1 hour. mRNA was isolated using RNeasy purification kit (Qiagen, Valencia, CA) and reverse transcriptase polymerase chain reaction (RT-PCR) was performed using OneStep RT-PCR kit (Qiagen) with human VEGFA primer pair (BioChain Institute, Inc., Hayward, CA) and GAPDH as a control. Quantitation was performed using the Kodak Image Station and image analysis software.

Cell Viability Assay

Cells were plated in 96 well plates at a density of 4×10^3 cells/well and allowed to attach overnight. The cells were washed with phosphate-buffered saline (PBS) and incubated in serum-free phenol red-free medium for 48 hours. Treatments were added as indicated in the figure legends for 72 hours. Cell viability was monitored using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega Corporation, Madison, WI) following the manufacturer's instructions. Absorbance values at 490 nm were recorded 1 hour after addition of the CellTiter 96 Aqueous One Solution Reagent using a Wallac Victor^2 1420 Multilabel Counter and readings were directly proportional to the amount of cellular proliferation. Control was set to 100 and all results were expressed relative to control.

In Vivo Tumor Xenograft Model

Female C.B.-17 scid 4- to 5-week old mice were obtained from Charles River (Wilmington, MA) and 201T lung tumor cells were harvested and suspended in sterile, serum-free PBS supplemented with 50% Matrigel (BD Biosciences, San Jose, CA). Cells (2×10^6) were injected in the hind flank region of each mouse, one site per mouse and allowed to grow. Six days after tumor implantation, the mice were divided into four treatment groups (10 mice per group): placebo, fulvestrant, vandetanib, and fulvestrant plus vandetanib. Treatment began 6 days after tumor implantation and lasted for 4 weeks. Fulvestrant (30 mg/kg) or vehicle control (peanut oil) was injected s.c. twice a week. Vandetanib (12.5 mg/kg) was administered daily by oral gavage at a volume of 0.2 mL/mouse. Tumor size was measured weekly and reported as an average relative tumor volume calculated as $(l \times w \times h \times \pi)/2$ (mm^3), where l is the length, w is the width, and h is the height of the tumor measured with calipers. At the end of the treatment period, the animals were killed and the tumors were removed and fixed in 10% buffered formalin for immunostaining. In a separate experiment, treatments were administered for 2 weeks and tumors were removed and frozen for protein isolation 2 hours after final treatment. Animal care was in strict compliance with the institutional guidelines established by the University of Pittsburgh.

Apoptosis Assay

The number of apoptotic cells was determined using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) as described previously.[25] Brown staining was considered positive. Slides were read and scored for the number of positive tumor cells per five high-powered fields per sample. Results are reported as the mean ± SE.

Immunohistochemistry and Immunofluorescence

Slides were deparaffinized with xylenes and rehydrated before heat-induced antigen retrieval. Nonspecific binding was blocked for 10 to 45 minutes at room temperature. Sections were incubated with primary antibodies for VEGFR-2 (1:50, Cell Signaling Technology, Clone 55B11), PECAM (1:100, Santa Cruz Biotechnology, Clone M-20), β-estradiol (prediluted from BioGenex, AR038-5R) as described,[26] ERβ (1:25, Santa Cruz Biotechnology, Clone H-150), P-ERβ (1:50, Abcam, ab62257), EGFR (1:500, DAKO, Clone H11), and VEGFA (1:100, Santa Cruz Biotechnology, Clone C-1) in PBS +0.5% BSA at 4°C overnight. For fluorescent staining, sections were incubated with conjugated fluorescent secondary antibodies at a 1:500 dilution (Donkey Anti-Goat 546 and Donkey-Anti-Rabbit 488, Invitrogen) for 1 hour at room temperature and staining was visualized using confocal microscopy (Lecia TCSSL). Immunoreactivity for immunohistochemistry was detected using biotinylated IgG secondary antibodies specific for each primary antibody followed by incubation with diaminobenzidine chromogenic substrate.

Statistical Analysis

Biochemical and animal data were analyzed using unpaired Student *t* test with or without Welch correction or analysis of variance as indicated in the figure legends (Graphpad Instat 3.06, GraphPad Software, Inc, San Diego, CA). All statistical tests were two sided with the threshold for statistical significance defined as $p < 0.05$.

RESULTS

NSCLC Cells Express 2 of the Targets of Vandetanib and VEGFA

Three NSCLC cell lines, 201T and A549 (derived from adenocarcinomas) and 273T (derived from a squamous cell carcinoma), were examined by immunoblot for expression of the vandetanib targets VEGFR-2, VEGFR-3, EGFR, and RET (Figure 1*A*). An appropriate positive control was used for each protein (VEGFA stimulated HUVEC for VEGFR-2, K562 erythroleukemia cells for VEGFR-3, A431 squamous cell carcinoma cells for EGFR, and TT thyroid medullary carcinoma cells for RET). All three NSCLC cell lines were positive for EGFR and VEGFR-3 and negative for VEGFR-2 and RET. VEGFR-2 negativity was confirmed by immunoprecipitation compared with positive control VEGFA-treated HUVEC cells. Ability of vandetanib to inhibit EGFR was shown by examining activation of MAPK in response to 10 ng/ml EGF, which we have previously documented is a rapid signaling response to EGFR activation in NSCLC cells.[17] Vandetanib produced a complete inhibition of phospho-MAPK after EGF (10 ng/ml) treatment in 201T, 273T, and A549 cells at a concentration of 2.5 μM or more (Figure 1*B*). Activation of Akt in response to 100 ng/ml VEGFC was used to determine if the kinase of VEGFR-3 was also blocked by vandetanib in these cells. A concentration of 5 μM vandetanib completely blocked the phosphorylation of Akt induced by VEGFC in 201T cells (Figure 1*C*). Complete inhibition of P-Akt was also observed in 273T and A549 cells (data not shown). Thus both EGFR and VEGFR-3 are potential targets of vandetanib in NSCLC cells.

Because VEGFR-2 is the major endothelial VEGFR responsible for angiogenesis in blood vessels, and is the most sensitive target of vandetanib, we also examined conditioned media from NSCLC cells for VEGFA, the main VEGFR-2 ligand (Figure 1*D*). Over time in nonconfluent culture, proliferating NSCLC cells secreted increasing amounts of VEGFA protein as detected by VEGFA-specific ELISA, up to 1200 pg/ml at 48 hours, after correcting for cell proliferation. Results are shown for 201T cells but similar VEGF production was also detected in 273T and A549 cells. Because VEGFR-3 responds to VEGFC and D, we also measured these VEGF ligands over time using specific ELISA assays. No VEGFD protein was detected in conditioned medium collected over a 48 hours period from any of the three cells lines. VEGFC secretion into conditioned medium was detected in these cell lines, and the amount secreted significantly increased over 48 hours after correcting for cell proliferation (Supplemental Figure 1) but was over 15-fold lower (75 pg/ml at 48 hours) than the amount of VEGFA produced. This suggests that there may be some autocrine stimulation of VEGFR-3 in these NSCLC cells, but the VEGFR-2 ligand VEGFA is the main form of VEGF secreted by NSCLC cells.

VEGFA Is an Estrogen-Responsive Gene in NSCLC

VEGFs are known to contain estrogen response elements and to be transcriptionally activated by estrogen in breast cancer.[22] We previously documented expression of ERs in NSCLC. Full-length ERβ mRNA and protein were found in all three of the lung cancer cell lines used here whereas full-length ERα was undetectable.[16] We examined relative expression of VEGFA mRNA and protein over time in the presence of 10 nM β-estradiol, a physiologic concentration, in 201T cells (Figure 2*A, B*). VEGFA mRNA detected by quantitative RT-PCR peaked at 1 hour after treatment with β-estradiol, and the 2.2-fold increase over baseline was significant at both 1 hour ($p = 0.001$) and 2 hours ($p = 0.0001$). The amount of VEGFA protein secreted into culture media, detected by ELISA, was also enhanced by β-estradiol 1.7-fold over control at 4 hours ($p = 0.0001$), and this effect persisted at 6 hours. Similar results in estrogen-induced VEGFA secretion were also observed in 273T and A549 cells (Supplemental Figure 2*A*). In contrast, we observed a small but not significant increase in VEGFC protein production after similar β-estradiol treatment, and VEGFD was still undetectable in conditioned medium from lung cancer cell lines after treatment with β-estradiol (data not shown). The ability of estrogen to increase production of VEGFA in lung cancer cells may produce heightened angiogenesis mediated by VEGFR-2 in endothelial cells in the tumor microenvironment, whereas VEGFR-3 activity controlled by VEGFC production by lung cancer cells may not be affected by estrogen.

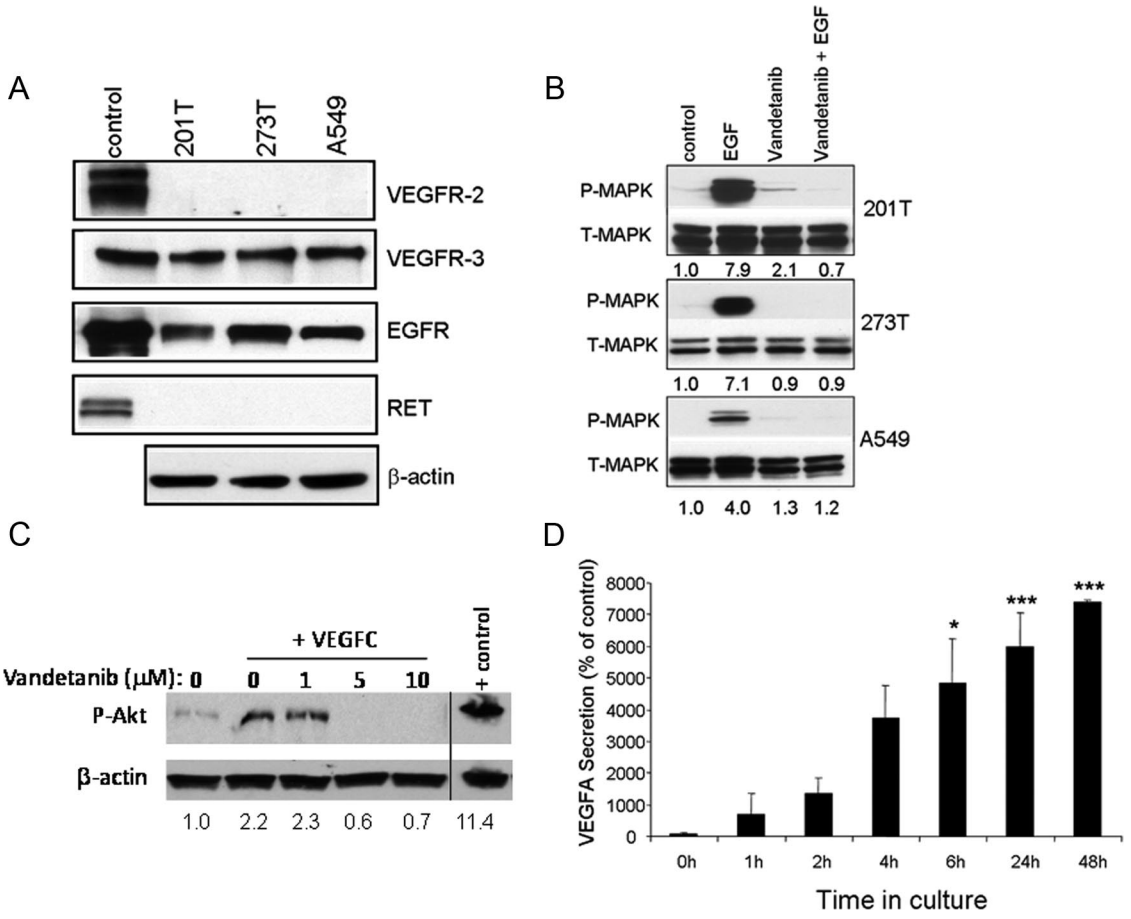

FIGURE 1. Protein expression in non-small cell lung cancer (NSCLC) cell lines and vascular endothelial growth factor (VEGF) secretion. *A*, Western blot analysis for VEGFR-2, VEGFR-3, epidermal growth factor receptor (EGFR), RET, and actin in whole cell lysates (50 μg) from NSCLC cells 201T, 273T, and A549. Cell line controls were specific for each protein of interest: VEGF stimulated HUVEC (VEGFR-2), K562 (VEGFR-3), A431 (EGFR), and TT (RET). *B*, 201T, 273T, and A549 cells were serum starved for 48 hours followed by treatment with 10 ng/ml EGF, 2.5 μM vandetanib or the combination followed by Western blot analysis for P-MAPK and T-MAPK. Representative images are shown and relative densitometric quantitation is shown below each blot. *C*, Vandetanib inhibits phosphorylation of Akt. Serum starved 201T cells were treated with VEGFC (100 ng/mL) for 30 minutes to induce downstream phosphorylation of Akt, followed by treatment with increasing concentrations of vandetanib. 201T cells treated with EGF (10 ng/ml) for 5 minutes was used for a positive control. *D,* VEGFA is secreted in lung tumor cells over time in culture. 201T cells were plated at time 0. Media was removed over time up to 48 hours and analyzed using VEGF Quantikine enzyme-linked immunosorbent assay (ELISA) kit. Data were normalized to total protein at each time point and are represented as relative secretion compared with control time 0. Results represent the mean ± SE per time point. *$p < 0.01$; ***$p < 0.0001$ analyzed by Student *t* test.

Vandetanib Inhibits ER-EGFR Cross-Talk in NSCLC

We have previously demonstrated that nongenomic signaling of β-estradiol, primarily through the ERβ, results in activation of proteases that cleave EGFR ligands such as TGF-α, which is followed by EGFR-driven activation of MAPK within 5 minutes.[17,19] This MAPK activation can be blocked by EGFR TKIs.[17] To determine if vandetanib can also block ER-EGFR cross-talk, we determined the phosphorylation status of MAPK after exposure to 10 nM β-estradiol in the presence and absence of vandetanib and compared it to inhibition with two positive controls, gefitinib and an EGFR neutralizing antibody, C225. Phosphorylation of MAPK was quantitated relative to total MAPK using densitometry and expressed as a percent of vehicle control (Figure 3*A*). Treatment with β-estradiol for 5 minutes resulted in a twofold induction of phospho-MAPK (significant compared with vehicle, $p = 0.0001$), which was significantly inhibited by 2 hours pretreatment with 10 μM vandetanib ($p = 0.0002$), comparable to the positive controls gefitinib and C225. Vandetanib alone was not significantly different from vandetanib plus β-estradiol. This shows that vandetanib can prevent activation of EGFR that is initiated by estrogen. This result was replicated in the other two cell lines (not shown). We also previously showed that long-term exposure to an EGFR TKI can induce ERβ protein expression in NSCLC, providing a feedback mechanism that could increase ERβ signaling during EGFR inhibition.[17] We determined that the multikinase inhibitor vandetanib also induced expression of

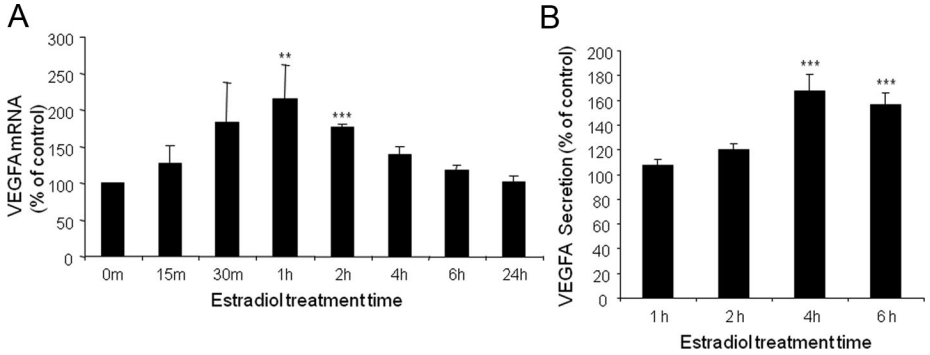

FIGURE 2. Estrogen induces vascular endothelial growth factor (VEGF) mRNA production and protein secretion. *A,* 201T cells were serum starved for 48 hours and then treated with 10 nM β-estradiol from 0 to 24 hours. mRNA was isolated using RNeasy purification kit and reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using OneStep RT-PCR kit with human VEGF primer pair. Results are the mean ± SE of two separate experiments each of which had two samples per time point. Control was set to 100. $^{**}p < 0.001$; $^{***}p < 0.0001$, Student *t* test. *B,* 201T cells were treated with 10 nM β-estradiol for 0 to 6 hours. Media was collected at each time point and analyzed using a Quantikine VEGF enzyme-linked immunosorbent assay (ELISA) kit. Total protein was isolated from each well and quantitated. Results were normalized for total protein and are expressed relative to the time 0 control. Results are representative of the mean ± SE of two separate experiments each of which had three samples per treatment group. $^{***}p < 0.0001$, Student *t* test.

ERβ protein in NSCLC (Figure 3*B*). ERβ detected by immunoblot increased fourfold over vehicle treatment at 4 days ($p = 0.02$) and sevenfold over vehicle treatment of 7 days ($p = 0.02$), after correction for amount of actin, following administration of 2.5 μM vandetanib. Blocking the EGFR with vandetanib may increase estrogenic signaling in NSCLC.

We then examined to what extent stimulation of VEGFA mRNA expression by β-estradiol occurred downstream of EGFR activation, because EGFR is known to transcriptionally regulate VEGF production,[27,28] and we previously found that nongenomic signaling of ERβ through EGFR activation predominated over nuclear signaling of ERβ through an estrogen response element in lung cancer cells.[16,19] Vandetanib and the positive control EGFR TKI gefitinib were used to inhibit EGFR before and during treatment with β-estradiol. Fulvestrant was also used to block ERβ, and the combined effect of fulvestrant and each TKI was also examined. Induction of VEGFA mRNA by β-estradiol was inhibited >75% by either gefitinib, vandetanib, or fulvestrant alone, and 100% (return to baseline) by the combination of either gefitinib or vandetanib with fulvestrant (Figure 3*C*). These findings suggest that ERβ nongenomic signaling to the EGFR is largely involved in VEGFA induction in NSCLC cells, although some VEGFA induction may occur through direct ER genomic signaling.

Combination Treatment with Vandetanib and Fulvestrant Shows Increased Antiproliferative Effects

We next determined the effect of combining vandetanib (at approximately the IC50 concentration for 201T and A549 cells determined from concentration-response curves) with the antiestrogen fulvestrant to increase the antiproliferative effect of vandetanib. Fulvestrant blocks the binding of β-estradiol to ERβ and we have previously shown has modest antiproliferative effects against NSCLC cells in culture as a single agent.[17] Cells were serum deprived and then stimulated to proliferate with a combination of β-estradiol and EGF as described in Materials and Methods. We have previously shown that β-estradiol enhances the proliferative effects of EGF.[19] In 201T and A549 cells (Figure 4), the combination of vandetanib and fulvestrant decreased relative cell proliferation in 201T cells from 43% of vehicle control with vandetanib alone to 26% of control with the combination ($p < 0.0001$). In A549 cells, relative cell proliferation decreased from 70% of control with vandetanib alone to 18% of control with the combination ($p < 0.0001$). Fulvestrant alone, which shows a maximum antiproliferative effect of 70% of control at the highest concentration that is soluble (20 μM), had only weak antiproliferative effects at the 5 μM concentration used for the combination study (83 and 96% of control in 201T and A549 cells, respectively). Similar results were also observed in 273T cells with the maximum inhibition observed with the combination treatment (Supplemental Figure 2*B*). Comparable enhancement of antiproliferative effects was observed when the two agents were combined at concentrations ranging from 1 to 5 μM (data not shown).

Vandetanib and Fulvestrant in Combination Maximally Inhibited Growth of NSCLC Xenografts

We confirmed the enhancement of vandetanib and fulvestrant in combination in an in vivo tumor growth experiment (Figure 5*A*). 201T cells were injected s.c. into immunocompromised mice and treatments were started after establishment of tumor xenografts at 7 days. Fulvestrant reduced mean tumor volume by 23% compared with vehicle control (n.s.), whereas vandetanib reduced mean tumor volume by 51% compared with vehicle ($p < 0.005$). The combination reduced mean tumor volume by 64% ($p < 0.000$l compared with vehicle, $p < 0.05$ compared with vandetanib alone, and $p < 0.005$ compared with fulvestrant alone). No toxicities, such as weight loss, loss of appetite, dehydration, or skin-

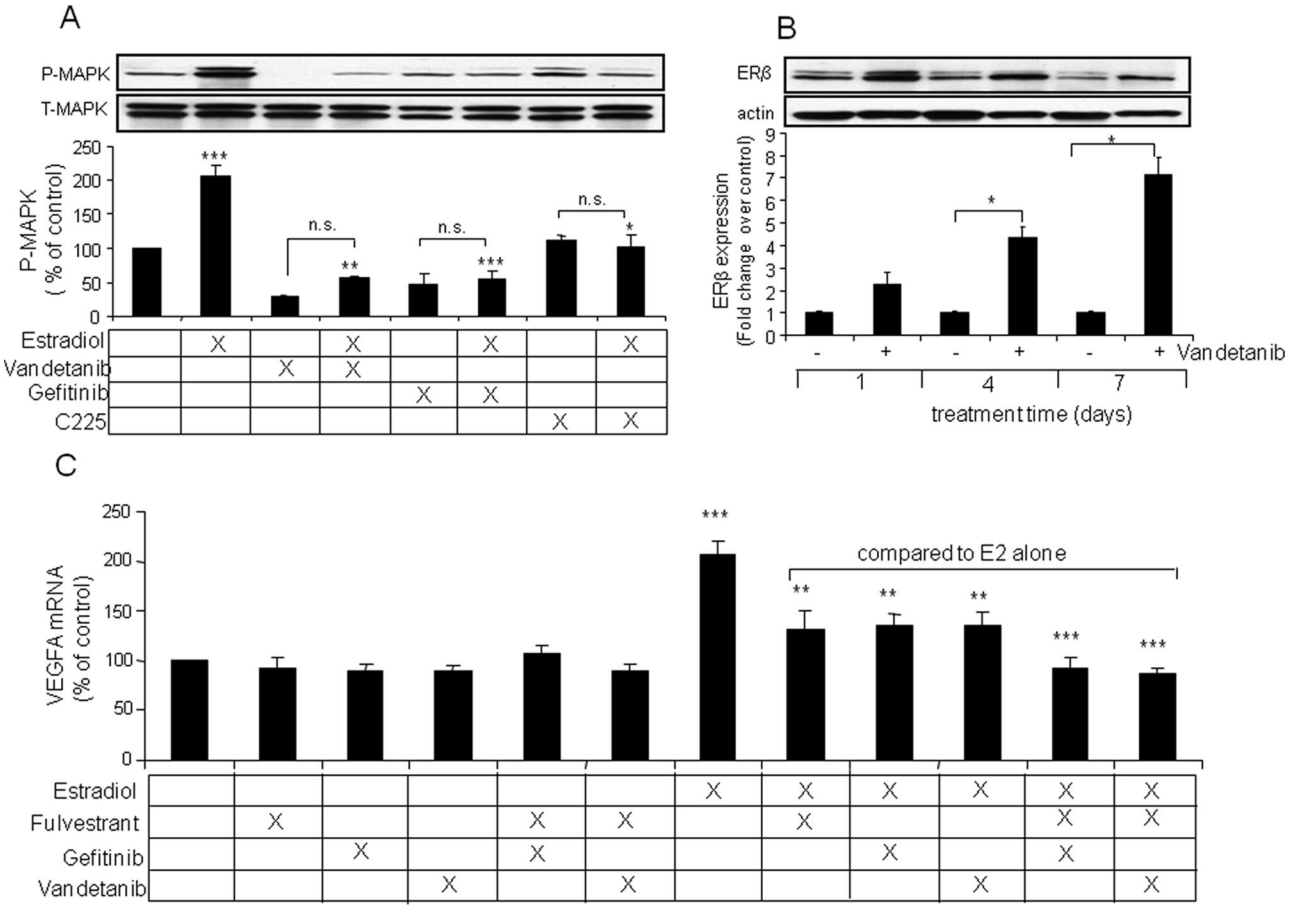

FIGURE 3. Vandetanib inhibits estrogen-induced P-MAPK and increased ERβ protein expression. *A*, Serum-deprived 201T cells were pretreated for 2 hours with vandetanib (10 μM), gefitinib (20 μM), and C225 (epidermal growth factor receptor [EGFR] blocking antibody, 6 μg/ml) or not treated at all followed by stimulation with 10 nM β-estradiol for 5 minutes; Representative Western blot. Each sample was analyzed by Western analysis using an anti-P-MAPK antibody and anti-T-MAPK antibody. No treatment was set to 100. Quantitative results represent the mean ± S.E. of results of four samples per experimental treatment. Unpaired Student *t* test, Welch corrected, compared with control, ***$p = 0.0008$. Treatment groups plus estrogen compared with estrogen alone, ***$p = 0.0001$, **$p = 0.0002$, *$p = 0.0039$, and n.s., nonsignificant. *B*, 201T cells were treated with 2.5 μM vandetanib or DMSO control in phenol-red free medium with 1% charcoal stripped serum for up to 7 days. A representative Western blot is shown of ERβ and actin expression. No treatment on day 1 was set to 1. Quantitation represents the mean ± S.E. of four samples per experimental treatment, normalized to actin control. Unpaired Student *t* test, Welch corrected, *$p = 0.02$. *C*, 201T cells were pretreated with gefitinib (20 μM), vandetanib (10 μM), or fulvestrant (5 μM) for 2 hours followed by treatment with 10 nM β-estradiol for 1 hour. mRNA was isolated and analyzed as in Figure *2A*. Results are the mean ± SE of two separate experiments each of which had two samples per time point. Control was set to 100. ***$p < 0.0001$, **$p < 0.001$.

related toxicities because of treatment were observed in the animals throughout the entire length of the experiment.

Extent of apoptosis in tumor xenografts was examined by immunohistochemistry of formalin-fixed excised tumors (Figure 5*B*); staining of apoptotic nuclei was increased by both single and combination treatments. The number of apoptotic cells was quantitated in sections from five tumors per treatment group (Figure 5*C*), showing a significant increase in apoptotic cells with combination treatment (mean of 32.5 cells/field) compared with vehicle (mean 2.5 cells/field, $p < 0.0001$), and compared with vandetanib alone (mean 22.5 apoptotic cells/field, $p < 0.01$), or fulvestrant alone (mean 14 cells/field, $p < 0.0001$). Single treatments were also significantly different from vehicle control (Figure 5*C*).

Confirmation that NSCLC In Vivo Express the Targets of Vandetanib and Fulvestrant

Xenografts were also examined to confirm expression of the targets of the agents used in vivo (Figure 6). Endothelial cells within lung tumor xenografts were positive for the endothelial cell marker PECAM (red, Figure 6*A*) and were also positive for the vandetanib target VEGFR-2 (green, Figure 6*A*). Dual-label immunofluorescence showed overlay of PECAM and VEGFR-2 staining (yellow, Figure 6*A*),

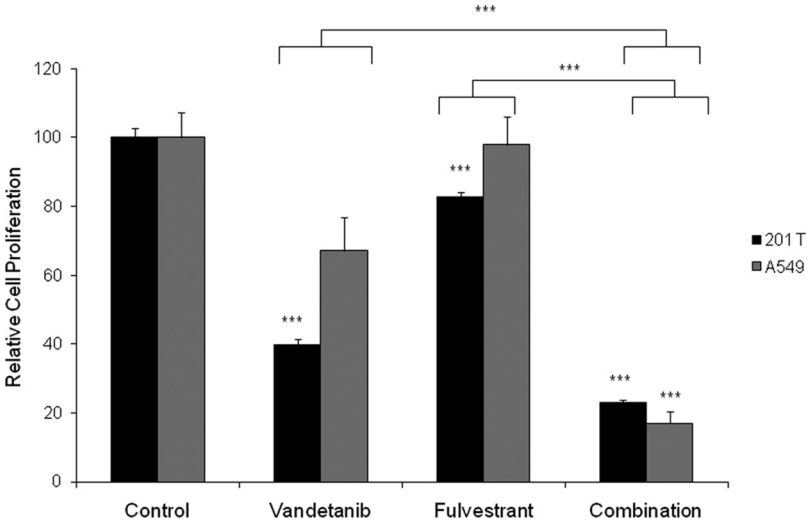

FIGURE 4. Effect of combined treatment of fulvestrant and vandetanib on cell proliferation. 201T and A549 cells were serum deprived for 48 hours followed by treatment with fulvestrant (5 μM) and/or vandetanib (1.25 μM) as indicated for 72 hours. All treatment groups received EGF (10 ng/ml) and β-estradiol (10 nM) for 72 hours. Cellular proliferation was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay. Cell Titer Reagent (20 μL) was added to each well and plates were incubated for 1 hour. Results represent the mean $\pm$ S.E. of 16 samples per experimental treatment. ***$p < 0.0001$, unpaired Student *t* test, Welch corrected, compared with control, and for fulvestrant and vandetanib treated data compared with the combination treated data.

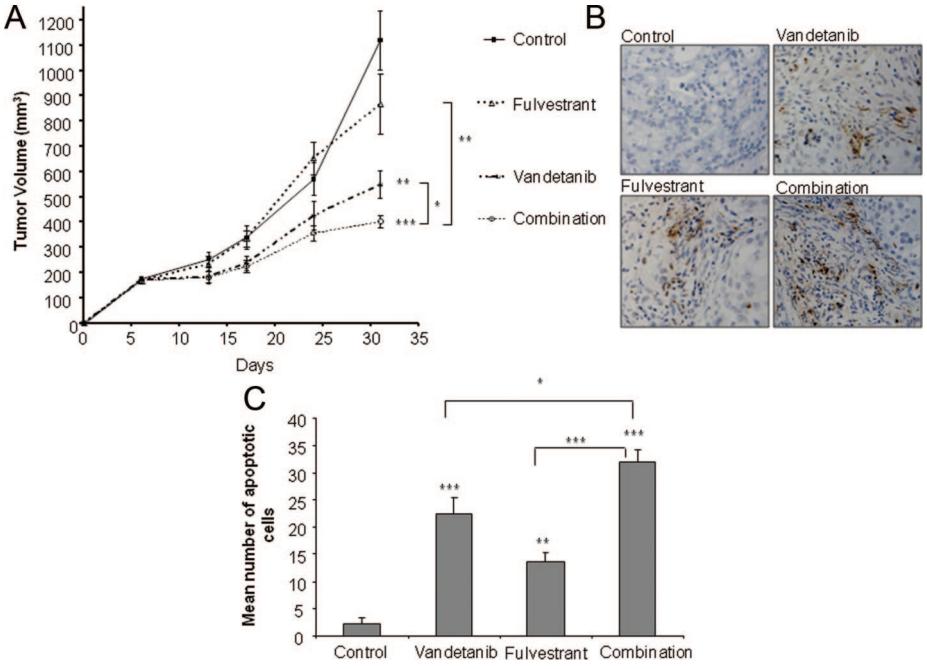

FIGURE 5. Combined fulvestrant and vandetanib treatment maximally inhibits tumor growth in mice. *A*, 201T tumor bearing mice received the following treatments for 4 weeks: vehicle control, fulvestrant (30 mg/kg), vandetanib (12.5 mg/kg), or combination. Treatment began 6 days after tumor implantation. Results represent the mean tumor volumes $\pm$ S.E. of 9 to 10 tumors per treatment group. Unpaired Student *t* test, Welch corrected. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0001$. *B*, Representative immunohistochemical images of apoptotic cells in tumors from different treatment groups at 20$\times$ magnification. *C*, Quantitative results. Positive cells were counted in five high-magnification areas per xenograft from five samples per treatment group. The results are presented as the mean number of apoptotic cells per area $\pm$ SE. Unpaired Student *t* test; *$p < 0.01$; **$p < 0.001$; and ***$p < 0.0001$.

demonstrating that VEGFR-2 protein is localized within tumor blood vessels.

Tumor cells within xenografts were also positive for two of the vandetanib targets, EGFR and VEGFR-3 (Figure 6*B*), whereas being negative for VEGFR-2 (not shown), as expected from immunoblots of cell cultures (Figure 1*A*). Tumors were also positive for ERβ (Figure 6*B*), as previously published,[17] and for its ligand β-estradiol suggesting active signaling (Figure 6*B*). Recently, Meireles et al.[26] has demonstrated that β-estradiol can be specifically detected in using immunohistochemical techniques. Two endpoints were used to demonstrate changes in ERβ-EGFR interaction following treatments: the amount of VEGFA produced and the presence of phospho-ERβ. ERβ can be phosphorylated by EGFR kinase signaling after which it is transported to the nucleus. Phosphorylated ERβ positive nuclei were observed from all treatment groups, with the least amount observed in the combination treatment group: control = 35.8 $\pm$ 4.3, fulvestrant = 28.6 $\pm$ 3.9, vandetanib = 23.0 $\pm$ 4.4, combination = 17.6 $\pm$ 4.8 positive nuclei per 200 mM area (control versus combination $p < 0.05$, all other comparisons n.s.). Xenografts were also positive for VEGFA (Figure 6*C*), the ligand for VEGR-2. Staining of VEGFA within xenografts was very diffuse, being present both within tumor cells and in the interstitial spaces, consistent with it being a secreted protein. A semiquantitative scale was therefore used to compare VEGFA staining among different treatments, to determine if inhibitors reduced VEGFA content, as predicted from the cell culture experiments. Below each image is the relative value of the overall VEGFA staining in each group, scored on a

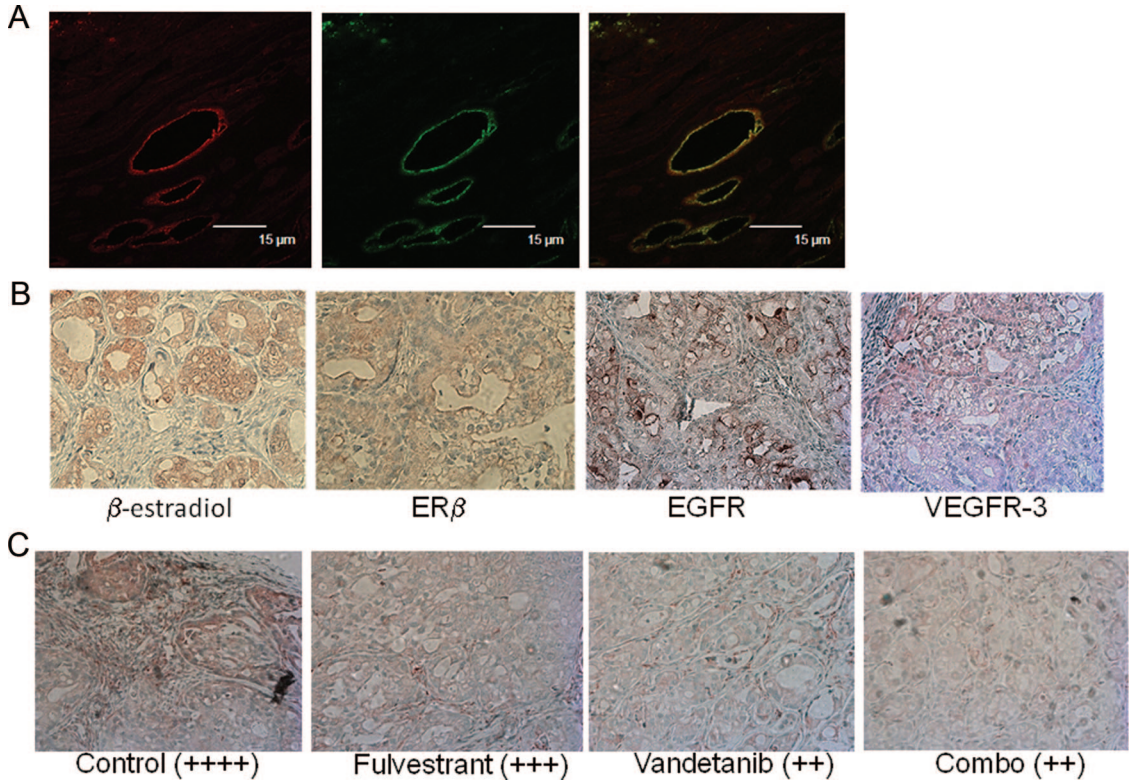

FIGURE 6. Expression of vandetanib and fulvestrant targets in tumor cells or blood vessels within tumor xenografts. *A*, Representative image of VEGFR-2 expression in endothelial cells. Dual-label immunofluorescence staining was performed on tumor xenograft tissue sections for the detection of VEGFR-2 (green) and PECAM (red). Colocalization of VEGFR-2 with endothelial cells is seen in the merged image. Areas of yellow represent colocalization. *B*, Representative immunohistochemical staining of β-estradiol, ERβ, epidermal growth factor receptor (EGFR), and vascular endothelial growth factor receptor (VEGFR)-3 in lung tumor xenografts. *C*, VEGFA is reduced by treatment in lung tumor xenografts. Representative staining from each treatment group is shown. Below each image is the relative value of the overall VEGFA staining in each group, scored on a scale of +1 to +5, +1 being staining observed in fewer than 10% of cells with low intensity and +5 being staining observed in 90% or more of cells and most intense staining.

scale of +1 to +5, +1 being staining observed in fewer than 10% of cells with low intensity and +5 being staining observed in 90% or more of cells and most intense staining. Treatment with the antiestrogen fulvestrant reduced relative staining for VEGFA compared with vehicle (Figure 6*C*, second panel). VEGFA staining was reduced to a greater extent by treatment with vandetanib and also by the combination (Figure 6*C*, two right panels), suggesting that loss of VEGFA is involved in the antitumor effects of both fulvestrant and vandetanib. Positive and negative control staining is shown in Supplemental Figure 3. Evidence that ER-EGFR cross-talk was also affected was seen by a decrease in the extent of phospho-MAPK labeling in xenografts with combination treatment (not shown). In combination treatment, inhibition of ERβ and EGFR interaction is likely to be involved in the enhancement by fulvestrant of antitumor effects of vandetanib.

Targeted Agents Modulate Signaling within Xenografts

To show that cell signaling is modulated within the xenografts after short-term exposure to targeted agents, in a separate experiment protein lysates were prepared from lung tumor xenografts 2 hours after the last treatment and analyzed for phospho-Akt and phospho-MAPK (Supplemental Figure 4). Three individual tumors from each treatment group were analyzed. Phospho-Akt and phospho-MAPK expression were decreased in each individual treatment group with the lowest expression in the tumors from the combination treatment group (61% inhibition of phospho-Akt and 64% inhibition of phospho-MAPK with combination treatment).

DISCUSSION

Combining targeted therapies in specific lung cancer patients with predicted sensitivity may increase efficacy of these agents. Understanding which oncogenic targets are present in lung cancer and how these pathways interact may also help to individualize drug therapy. Here, we show that the antitumor effect of the multikinase inhibitor vandetanib can be enhanced by addition of the antiestrogen fulvestrant, a drug that is well tolerated and has shown activity in treatment of ER-positive breast cancer.[29–31] Enhanced antitumor effects were observed in cell culture with isolated NSCLC cells that

are ERβ positive[16] and in a xenograft model containing tumor vasculature. We showed that in NSCLC cell lines that are positive for several targets of vandetanib (EGFR and VEGFR-3 and the VEGFR-3 ligand VEGFC) increased antiproliferative effects can be achieved in cell culture with the combination of vandetanib and fulvestrant. VEGFA, an important ligand for VEGFR-2 (the major VEGFR family member found in endothelial cells), was also found to be released by NSCLC cells under the control of the ER pathway and enhanced VEGFA secretion appeared to be mainly downstream of an estrogen-dependent activation of EGFR, which could be maximally inhibited by combining agents targeting EGFR and ER. In tumor xenografts, an increased antitumor effect of these two inhibitors was also observed. The presence of VEGFR-2 on vasculature in the tumor microenvironment suggests that this additional target for vandetanib, which can respond to VEGFA released by β-estradiol stimulation may also be relevant to the increased antitumor effect. Lung tumor xenografts were found to express both ERβ and β-estradiol. We have demonstrated previously that ERβ is found both in the cytoplasmic compartment and the nuclear compartment in lung tumors and that cytoplasmic ERβ protein expression is an independent negative predictor of overall survival in NSCLC, consistent with the cytoplasmic ERβ staining observed here.[19,21] We have also found that the phosphorylated form of ERβ is mainly nuclear and is maximally decreased by the combination. Using fulvestrant and vandetanib together to inhibit ER-EGFR cross-talk may result in a greater reduction in proliferation and a reduction in the activity of the VEGFA-VEGFR2 pathway involved in angiogenesis. The ability of vandetanib to inhibit VEGFR-3 expressed by tumor cells may also contribute to its antitumor effects.

Vandetanib has recently shown promise in the treatment of medullary thyroid cancer, a disease in which the RET gene, a key target of vandetanib, is frequently mutated or over-expressed through gene amplification.[13,14,32] Vandetanib showed some promising results in phase II trials in lung cancer, but randomized phase III trials in combination with other agents such as pemetrexed and docetaxel did not show increased overall survival, as summarized in a recent report.[15] Nevertheless, there may be a subset of lung cancer patients that might respond to vandetanib combinations, particularly if the combination inhibited complementary signaling targets. Recent findings from the Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) trial shows that vandetanib treatment was beneficial in patients with EGFR mutation/amplification or VEGF/VEGFR2 expression.[33] Our preclinical data support a possible role for a combination of vandetanib with an antiestrogen such as fulvestrant to achieve greater antitumor effects in lung tumors expressing ERβ, β-estradiol, VEGFR-2, VEGFA, and EGFR. An EGFR mutation that sensitizes to erlotinib or gefitinib was not required to see an enhanced effect. An increased antitumor effect was observed using cells lacking an EGFR mutation that sensitizes the receptor to EGFR TKIs. The mechanism of increased antitumor effect seems to involve inter-related ER-EGFR signaling and modulation of VEGFR-2 signaling.

A concern with vandetanib is the toxicity profile, which includes diarrehea, rash, acne, nausea, hypertension, headache, and rare but potentially dangerous prolongation of the QT interval.[14] Fulvestrant can be given for long periods and can exhibit toxicities relating to withdrawal of estrogen, such as hot flashes.[29–31] Because fulvestrant and vandetanib have nonoverlapping toxicities, fulvestrant would not be expected to increase the toxicity of vandetanib. Our previous phase I study showed no increased toxicity of gefitinib in combination with fulvestrant in advanced lung cancer.[20]

Many studies have now demonstrated that estrogen receptors are frequently expressed in NSCLC,[33,34] along with the enzyme aromatase,[21,35,36] resulting in high intratumoral concentrations of β-estradiol.[37] ERs, especially ERβ probably mediate several protumor effects in lung cancer including increased proliferation, reduced apoptosis, and increased angiogenesis. The gene for VEGFA, a major angiogenic protein, is known to contain an ERE that can mediate ER-induced gene transcription directly.[22] In addition, EGFR signaling through Akt is known to lead to induction of VEGFA transcription,[27] probably involving activation of the transcription factor Sp1.[38] Direct stimulation by estrogen of VEGFA transcription by means of an ERE or indirect stimulation by means of ER-EGFR cross-talk are both possible in NSCLC cells, although our data support a major role for nongenomic activation of EGFR in mediating this effect. Fulvestrant and vandetanib may limit the amount of VEGFA that is available in addition to having a direct effect to block a group of receptors important for stimulating tumor growth.

One of the disappointments of EGFR TKIs is their relatively low activity against lung cancer that lacks an EGFR sensitizing mutation. Combination therapy might be a strategy to improve responses in these cases, which represent the majority of lung cancer. Selection of patients with high tumor expression of several targets of vandetanib, and ligands for these targets, and coexpression of ERβ and β-estradiol might improve the likelihood of seeing clinical responses to the combination of vandetanib and an antiestrogen such as fulvestrant. Because ERβ cross-activates EGFR and each of these receptors seems to be induced when the other is inhibited (as shown in Ref. 17 and data presented here), the combination of vandetanib and fulvestrant might also prevent induction of a compensatory mechanism that could limit effectiveness of either compound alone.

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