Contrary Effects of the Receptor Tyrosine Kinase Inhibitor Vandetanib on Constitutive and Flow-Stimulated Nitric Oxide Elaboration in Humans

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Abstract—Vascular endothelial growth factor regulates neoplastic angiogenesis through production of endothelium-derived NO. We performed a prospective evaluation of vascular function during treatment with vandetanib, a vascular endothelial growth receptor 2 and 3 receptor tyrosine kinase inhibitor, to determine the effects of vascular endothelial growth receptor signal interruption on endothelial function in humans. Seventeen patients with stage IV breast cancer received dose-escalated vandetanib in combination with low-dose oral chemotherapy. We measured blood pressure, systemic nitrate/nitrite levels, and brachial artery vascular function. In vitro analyses of cultured endothelial cells were performed to determine the effect of vandetanib on NO production, akt473 phosphorylation, and endothelial NO synthase protein content and membrane localization. Vandetanib treatment for 6 weeks significantly increased blood pressure, decreased resting brachial artery diameter, and decreased plasma systemic nitrate/nitrite levels compared with baseline. Flow-mediated vasodilation was preserved, and no change was noted in nitroglycerin-mediated vasodilation. In vitro, endothelial cell nitrite levels and akt473 phosphorylation were reduced and vascular endothelial growth receptor 2 levels did not change, but endothelial NO synthase membrane concentration doubled. Vandetanib reduces constitutive NO production and increases blood pressure, yet flow-stimulated NO bioavailability was preserved. Changes in vascular function with tyrosine kinase inhibition are complex and require further study in humans. (Hypertension. 2011;58:85-92.)

Key Words: vascular endothelial growth factor ■ VEGF receptor 2 ■ secondary hypertension ■ endothelium ■ NO

Angiogenesis is an obligate component of the growth and progression of many solid tumors, including breast cancer.1–3 Vascular endothelial growth factor (VEGF), a potent angiogenic factor that regulates malignant angiogenesis, has emerged as a therapeutic target for this disease. Angiogenesis inhibition using the anti-VEGF antibody bevacizumab in combination with cytotoxic chemotherapy has improved response rate and prolonged progression-free survival in patients with advanced cancer.4 Small molecule tyrosine kinase inhibitors of the VEGF receptor (VEGFR) are under investigation as antiangiogenesis therapy for breast cancer.5–7

Hypertension is a prevalent adverse effect of VEGF inhibitors8–12 and VEGFR antagonists13–16 and quite distinct from chemotherapy-related toxicity. The incidence ranges from 15% to 60%, depending on the antiangiogenic agent, tumor type, and patient-related factors.17 Indeed, nearly all patients experience some increase in blood pressure, if not frank hypertension.18 The mechanisms underlying hypertension associated with angiogenesis inhibitors are not well defined. Several theories have been proposed, including an imbalance in neurohumoral factors and the development of vascular rarefaction. In humans treated with the VEGFR2 inhibitor sorafenib with a mean increase in blood pressure of 9 mm Hg, there was no change in renin, aldosterone, and endothelin 1 levels, whereas catecholamine levels trended down inversely to the increase in blood pressure,15 making humoral changes less likely.

The most compelling explanation for angiogenesis inhibitor-associated hypertension is its effect on NO bioavailability described in preclinical investigations. VEGF is known to stimulate endothelial NO synthase (eNOS), leading to NO production and vasodilation.19,20 Inhibition of VEGF signaling could reduce eNOS activity and decrease NO levels, leading to vasoconstriction, endothelial dysfunction, and subsequent hypertension. Animal data show that VEGFR inhibition leads to reduced eNOS activity and hypertension.21

The effect of VEGF inhibitors on NO bioavailability and its role in treatment-related hypertension in humans is not well described. Accordingly, we evaluated blood pressure, markers of NO production, and in vivo vascular function before and during exposure to the VEGFR 2/3 antagonist, vandetanib, in subjects participating in a trial of vandetanib and metronomic chemotherapy in advanced metastatic breast cancer to test constitutive and flow-stimulated NO bioavailability.

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Methods

Subjects
Eligible subjects with metastatic breast cancer were recruited as part of a single-center investigator-initiated phase 1 trial. The subjects had exposure to ≥4 previous chemotherapy regimens for metastatic disease, an Eastern Cooperative Oncology Group performance status of ≥2, were postmenopausal or surgically sterile, and had normal bone marrow, hepatic, and renal function. Patients were required to have preserved cardiac function (left ventricular ejection fraction >45%), no evidence of QTc prolongation, and adequately controlled blood pressure at baseline. Patients were excluded for a requirement of >1 medication to control blood pressure to <140/90 mm Hg, therapeutic anticoagulation, or clinically significant cardiac disease.

Study Design
Three sequential cohorts, planned enrollment 8 patients each, were treated with escalating doses of daily vandetanib, an oral inhibitor of VEGFR and epidermal growth factor receptor (cohort 1: 100 mg; cohort 2: 200 mg; cohort 3: 300 mg), with continuous metronomic chemotherapy (cyclophosphamide, 50.0 mg PO once daily, and methotrexate, 2.5 mg PO twice daily, days 1 and 2 of each week). Vandetanib is a potent VEGFR2 inhibitor (inhibitory concentration22: 40 nmol/L) and shows additional inhibitory activity against rearranged during transfection receptor (IC50 100 nmol/L), Fms-related tyrosine kinase 4/VEGFR3 (IC50 110 nmol/L), and epidermal growth factor receptor (IC50 500 nmol/L).23-24

Subjects underwent vascular function testing before administration of vandetanib and after 6 weeks of protocol therapy. Because there were no differences in change in blood pressure or vascular function between cohorts, all of the subjects were studied as one group. All of the participants provided written informed consent. The protocol was approved by the human research committees of the Brigham and Women’s Hospital and Dana Farber Cancer Institute.

Blood Pressure Assessments
Blood pressure measurements were performed at each vascular study using an oscillometric device after 10 minutes of quiet recumbency before vascular measurements. The blood pressures were measured in triplicate with the value provided the average of the 3. Blood pressure was measured before vascular studies. All of the studies were performed in the morning after an overnight fast. Antihypertensive medications were held the day of study.

Assessment of Vascular Function
To assess endothelium-dependent, NO-mediated conduit artery vasodilation, brachial artery flow-mediated vasodilation after a hyperemic stimulus was measured at baseline and after 6 weeks on protocol therapy. The flow-mediated vasodilation technique was performed according to published protocol.25 We and others have demonstrated that brachial artery vasodilation 1 minute after reactive hyperemia dilation is mediated by NO.26,27

Subjects were studied in a controlled environment in the supine position after a minimum 4-hour fast. After a minimum 10-minute equilibration period, baseline 2D images of the brachial artery were obtained ≥2 cm above the antecubital fossa. A blood pressure cuff, placed proximal to the imaging transducer on the upper arm, was inflated to suprasystolic pressure for 5 minutes. The vessel was imaged continuously for 70 seconds after release of occlusion. Longitudinal brachial artery digital images from end-diastole (peak of the QRS) were acquired with a high-resolution (7.5 MHz) linear-array vascular ultrasound scanning probe (Vivid 7, GE Medical Systems, Piscataway, NJ). Reactive hyperemia was confirmed by measuring arterial blood flow using pulse-wave Doppler scanning interrogation. After a 10-minute period of re-equilibration, baseline measurements were repeated. Endothelium-independent vasodilation was assessed by measuring brachial artery diameter under basal conditions and 3 minutes after the administration of sublingual nitroglycerin (0.4 mg). Nitroglycerin was not administered if systolic blood pressure was <100 mm Hg. Subjects in whom nitroglycerin was not contraindicated were administered 0.4 mg of nitroglycerin sublingually, and the brachial artery was imaged continuously for 5 minutes as described above.

Blinded measurement of vessel diameter was performed using customized image analysis software (Brachial Analyzer for Research 5.7.9, Medical Imaging Applications, Iowa City, IA) by investigators blinded to study day and subjects (W.M.R. and J.A.B.). For each condition, the results from 6 images at end-diastole were averaged. Flow-mediated vasodilation was defined as the brachial artery diameter 60 to 70 seconds after cuff deflation compared with the baseline vessel diameter. Nitroglycerin mediated dilation was defined as the brachial artery diameter 3 minutes after administration of nitroglycerin compared with the baseline vessel diameter. To be considered interpretable, a study had to have distinct visualization of the proximal and distal intima-media arterial layers perpendicular to the ultrasound beam with <5% diameter variation across the field of measurement. In our laboratory, the intraobserver variability in measuring brachial diameters was 2.9%, and the variability of the hyperemic response was 1.4%.28

Volumetric flow, Q, was calculated from commercially available software (Brachial Analyzer, Medical Imaging Applications) by integrating the area under the velocity spectral waveform and dividing by the time required to arrive at a time-averaged velocity. Mean flow was calculated by multiplying the time-averaged velocity by the mean area of the lumen (as obtained from the index segment M-mode measurements). Mean shear stress was calculated according to the Hagen-Poiseuille formula, \( \tau_s = 4 \eta Q / \pi r_s^3 \), where \( \tau_s \) is shear stress in dynes per centimeter squared and mean volumetric flow is Q. The viscosity of blood, \( \eta \), is assumed to be 0.035 poise. The lumen radius, \( r_s \), is in centimeters. Forearm vascular resistance was calculated by dividing the mean arterial pressure by mean volumetric flow (arbitrary units).

Laboratory Analyses
The effects of angiogenesis inhibitor therapy on NO and reactive oxygen species production were examined in patients. Plasma samples were collected at baseline and after 6 weeks of treatment and frozen until use.

NO Assay
A quantitative fluorometric extracellular NO assay (Calbiochem) was used to assess NO production in patient samples following the manufacturer’s instructions. Because the final products of NO are nitrates and nitrites, this assay uses nitrate reductase to convert nitrates to nitrites, then uses 2,3-diaminonaphthalene and NaOH to convert nitrites to a fluorescent compound to measure total NO production. Plasma samples were thawed, filtered using Ultracel 10 kDa cutoff Microcons (Millipore) by centrifugation (0.5 hours, 4°C), and processed in the NO assay. Plates were read using a Wallac 1420 Multilabel Victor 3 microplate reader (Perkin Elmer), with excitation at 355 nm and emission at 430 nm.

DNA Damage ELISA
The marker 8-hydroxy-2’-deoxyguanosine is an oxidized nucleoside excised from damaged DNA that increases in the setting of oxidative DNA damage. Levels of 8-hydroxy-2’-deoxyguanosine were quantified in patient plasma samples using the colorimetric DNA Damage ELISA (Assay Designs) as per the manufacturer’s instructions. Absorbances at 450 nm were measured using a Spectra Max 190 microplate reader with SoftMax Pro software (Molecular Devices).

Nitrotyrosine ELISA
Superoxides are highly reactive and can react with NO to form peroxynitrite, which reacts with tyrosines on proteins causing tyrosine nitration (tyrosine converted to 3-nitrotyrosine). Patient plasma samples were examined using a quantitative chemiluminescent nitrotyrosine ELISA to measure tyrosine nitration as per the manufacturer’s instructions (Millipore). Luminescence was measured using a Wallac 1420 Multilabel microplate reader.
In Vitro Endothelial Cell Studies

Mile Sven 1 (MS1) endothelial cells (ECs) were cultured as we described previously.29

NO Assay
Vandetanib or matched vehicle control (dimethyl sulfoxide [DMSO]) was added to MS1 EC (n=6 per group with studies done in duplicate) in phenol red-free DMEM/0.5% BSA and incubated (0.5 hours, 37°C, 10% CO2). Then, 100 μmol/L of L-arginine and 100 μmol/L of soluble N-ethylmaleimide sensitive factor attachment proteins were added, cells were incubated (1.5 hours, 37°C, 10% CO2), and the NO assay was performed as described above.

Akt Activity
The MS1 ECs were cultured in 24-well plates in low-glucose (1.0 g/L) DMEM (LG-DMEM) supplemented with 10% FCS/1% l-glutamine-penicillin-streptomycin (37°C, 10% CO2) and grown to near confluence. Cells were rinsed twice in LG-DMEM/0.5% BSA. In some assays, cells were serum-starved in LG-DMEM/0.5% BSA (2 hours, 37°C, 10% CO2). Vandetanib (Selleck/Husker Chemicals) was dissolved in DMSO and diluted in LG-DMEM/0.5% BSA. Either 1 μmol/L of vandetanib or matched vehicle control was added to cells and incubated (1 hour, 37°C, 10% CO2). Protein lysates were prepared, and phosphorylation of aktS473 was measured as we described previously.29 Films were scanned, and Scion Image software was used to conduct densitometric analyses to quantify signal intensity (n=4 to 6 per group, studies done in triplicate).

eNOS Protein Levels in Membrane and Cytosol
The MS1 ECs were cultured in their normal culture medium and grown to confluence. Cells were rinsed in PBS and were serum starved in culture medium diluted 1/5 with LG-DMEM (2 hours, 37°C, 10% CO2). Vandetanib was dissolved in DMSO, and either 1 μmol/L of vandetanib or DMSO vehicle control was added to cells and incubated (1 hour, 37°C, 10% CO2). Protein lysates were prepared, and membrane and cytosol components were isolated using the Subcellular Protein Fractonation kit (Pierce). Both membrane and cytosolic fractions were run on gels and Western blotting conducted as we described.29 eNOS (rabbit monoclonal antibody, clone 49G3 at 1/1000), GAPDH (horseradish peroxidase–conjugate rabbit monoclonal antibody, clone 14C10 at 1/5000), and vascular endothelial (VE)-cadherin (rabbit polyclonal antibody, clone H72 at 1/1000) proteins levels were measured; films were scanned; and Scion Image software was used to conduct densitometric analyses to quantify signal intensity (n=3 per group, studies done in duplicate).

Statistical Considerations
Descriptive measures are reported as mean±SD. Experimental measures are reported as mean±SE. Biomarkers are reported as median (interquartile range). Basal forearm blood flow and diameter were compared by paired 2-tailed t tests. Laboratory measures were compared by Mann-Whitney U testing for 2-way comparisons. Association between vascular function and blood pressure changes were correlated by Pearson correlation. Statistical significance was accepted at the 95% confidence level (P<0.05). SPSS (version 16.0, SPSS Inc, Chicago, IL) software package was used.

Table 1. Subject Demographics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Data (N=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>49 (29 to 71)</td>
</tr>
<tr>
<td>Cardiovascular risk factors, %</td>
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<tr>
<td>Postmenopausal</td>
<td>100</td>
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<tr>
<td>Hypertension</td>
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</tr>
<tr>
<td>Dyslipidemia</td>
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<tr>
<td>Current smoker</td>
<td>0</td>
</tr>
<tr>
<td>Eastern Cooperative Oncology Group</td>
<td>0 (0 to 2)</td>
</tr>
<tr>
<td>performance status (median: 19)</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive medications, n</td>
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<tr>
<td>β-adrenergic blockers</td>
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<tr>
<td>Lisinopril</td>
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</tr>
<tr>
<td>Furosemide</td>
<td>1</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>1</td>
</tr>
<tr>
<td>Previous systemic therapy, %</td>
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<tr>
<td>Anthracycline exposure</td>
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</tr>
<tr>
<td>Trastuzumab or lapatinib</td>
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</tr>
<tr>
<td>Bevacizumab</td>
<td>41</td>
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<tr>
<td>Cetuximab</td>
<td>12</td>
</tr>
<tr>
<td>Cediranib</td>
<td>6</td>
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</tbody>
</table>

Results
Twenty-three patients were enrolled into the phase 1 trial. Nineteen subjects consented to participate in the vascular study, and 2 withdrew consent midprotocol. Patient and tumor characteristics are described in Table 1 for the 17 subjects who completed the investigation. All of the patients enrolled received protocol therapy.

Blood Pressure and Constitutive NO Bioavailability
Consistent with previous studies of VEGF receptor tyrosine kinase inhibitors (RTKIs), vandetanib increased mean arterial pressure from 91±8 to 102±10 mm Hg (P=0.001; Table 2). The increase in blood pressure was similar in both systolic and diastolic components. In contrast, no change in heart rate was noted. Also, subjects’ weight decreased significantly. Two laboratory measures of NO were used to determine NO bioavailability before and during vandetanib use. Nitrogen oxide (NOx) levels decreased significantly from baseline after exposure to study drugs for 6 weeks (Figure 1; P=0.036), indicating reduced NO elaboration. There was a trend between the reduction of nitrite level and increase in blood pressure (R2=0.21; P=0.068). When divided by the median blood pressure increase (10 mm Hg), the subjects with an increase of more than the median increased mean arterial pressure by 19±6 mm Hg compared with the subjects with an increase of less than the median, who had a nonsignificant change of 1±5 mm Hg (P<0.001). The subjects with an above-the-median increase in blood pressure had a 10-fold greater reduction in NOx compared with the 9 subjects whose blood pressure was below the median (−3945±5530 versus −382±2492; P=0.048). Levels of nitrotyrosine trended lower from baseline after 6 weeks of treatment (Table 2; P=0.054). Physiological evidence of reduced NO was evaluated with brachial ultrasonography. Baseline arterial diameter was 2.74±0.09 mm and decreased after 6 weeks of treatment to 2.57±0.08 mm (P=0.004; Table 2). Consistent with a reduction in NOx and increased blood pressure, forearm vascular resistance increased from 5.3±0.5 to 6.4±0.5 U (P=0.015). Shear stress did not vary significantly between study conditions (10.9±1.2 versus 11.6±1.7 poise; P=0.4).

We investigated the putative mechanism of reduced constitutive NO bioavailability in MS1 ECs. Incubation of vandetanib with MS1 EC significantly decreased nitrite production compared with the matched vehicle (DMSO;
Vandetanib lowered nitrite levels (**) after 6 weeks (\(P = 0.0003\)) in MS1 ECs. VEGF was used as a positive control and increased nitrite levels (**\(P = 0.02\)). These findings indicate that vandetanib lowered endothelial cell NO levels.

**Vascular Function Studies and Stimulated NO Bioavailability**

We measured flow-mediated vasodilation as an index of flow-stimulated NO bioavailability. The application of a phosphorylation of the intermediate, AKT at serine 473. Western blotting (Figure 3) shows that vandetanib also reduced phosphorylation of Akt at serine 473 in MS1 ECs compared with vehicle controls (DMSO; Figure 3; \(P = 0.01\)), indicating reduced Akt activation. Oxidative stress, as a mechanism to reduce NO bioavailability, was also evaluated. Levels of 8-hydroxy-2'-deoxyguanosine, a product of oxidative DNA damage, were stable with treatment (Table 2) and did not vary by vandetanib dose levels. In addition, nitrotyrosine levels trended lower during treatment. This may suggest a decrease in oxidative stress or a reduction in circulating nitrates from which to nitrosylate proteins.

**Table 2. Experimental Measures**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>6 wk</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>138±20</td>
<td>150±21</td>
<td>0.037</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>68±7</td>
<td>77±8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>91±8</td>
<td>102±10</td>
<td>0.001</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>74±13</td>
<td>71±10</td>
<td>0.28</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>72.8±17.0</td>
<td>71.3±16.5</td>
<td>0.004</td>
</tr>
<tr>
<td>Basal brachial artery diameter, mm</td>
<td>2.74±0.09</td>
<td>2.57±0.08</td>
<td>0.004</td>
</tr>
<tr>
<td>Post-RH brachial artery diameter, mm</td>
<td>3.07±0.12</td>
<td>2.93±0.12</td>
<td>0.06</td>
</tr>
<tr>
<td>Absolute increase in brachial artery diameter, mm</td>
<td>0.33±0.05</td>
<td>0.36±0.06</td>
<td>0.36</td>
</tr>
<tr>
<td>Reactive hyperemic stimulus, fold increase</td>
<td>5.8±2.9</td>
<td>6.8±3.0</td>
<td>0.024</td>
</tr>
<tr>
<td>Flow-mediated vasodilation, %</td>
<td>12.0±1.7</td>
<td>13.8±1.8</td>
<td>0.15</td>
</tr>
<tr>
<td>Prentiglycerin-brachial artery diameter, mm</td>
<td>2.78±0.1</td>
<td>2.63±0.1</td>
<td>0.024</td>
</tr>
<tr>
<td>Postnitroglycerin-brachial artery diameter, mm</td>
<td>3.38±0.12</td>
<td>3.16±0.13</td>
<td>0.04</td>
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<tr>
<td>Nitroglycerin-mediated vasodilation, %</td>
<td>22.5±2.0</td>
<td>20.3±2.3</td>
<td>34</td>
</tr>
<tr>
<td>Nitrates, AU</td>
<td>48 446±1264</td>
<td>46 799±1740</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nitrotyrosine, AU</td>
<td>259 814±26 139</td>
<td>246 157±20 501</td>
<td>0.027</td>
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<tr>
<td>8-Hydroxy-2'-deoxyguanosine, AU</td>
<td>1.96±0.09</td>
<td>2.0±0.15</td>
<td>0.5</td>
</tr>
</tbody>
</table>

AU indicates arbitrary units.

Figure 2; \(P = 0.0003\)). These findings demonstrate that vandetanib lowered EC NO levels. VEGF, used as a positive control, increased nitrite levels compared with matched vehicle (PBS; \(P = 0.02\)). We next evaluated the effect of vandetanib on VEGF to eNOS signaling by measuring

**Figure 1.** Vandetanib reduced plasma nitrite levels in humans. Plasma levels of nitrites were measured in patients at baseline and after receiving vandetanib plus chemotherapy for 6 weeks. Vandetanib lowered nitrite levels after 6 weeks (**\(P = 0.0001\)), suggesting that circulating levels of NO were reduced.

**Figure 2.** Vandetanib reduced extracellular nitrite levels in endothelial cells. MS1 endothelial cells (ECs) were incubated with 1 \(\mu\)mol/L of vandetanib or matched vehicle (dimethyl sulfoxide [DMSO]), 50 ng/mL of vascular endothelial growth factor (VEGF) or matched vehicle (PBS; 0.5 hours), and l-arginine and soluble N-ethylmaleamide sensitive factor attachment protein (SNAP) added (1.5 hours). Vandetanib lowered nitrite levels in MS1 ECs (**\(P = 0.0003\)). VEGF was used a positive control and increased nitrite levels (**\(P = 0.02\)). These findings indicate that vandetanib lowered endothelial cell NO levels.

**Figure 3.** Vandetanib reduced phosphorylation of Akt in endothelial cells (ECs). MS1 ECs were incubated with 1 \(\mu\)mol/L of vandetanib or matched vehicle (dimethyl sulfoxide [DMSO]; 1 hour). Western blotting analysis showed that vandetanib decreased phosphorylation of Akt (S473) in MS1 ECs (**\(P = 0.01\); \(n = 6\) per group, studies done in triplicate). These findings show that vandetanib reduced Akt activity.
5-minute ischemic intervention created a significant reactive hyperemic stimulus during both study visits. Peak reactive hyperemic blood flow increased 5.8±2.8-fold over basal flow at baseline and 6.8±3.0-fold at 6 weeks (P=0.02). In the whole cohort, flow-mediated vasodilation did not change significantly during the 6 weeks of treatment (12.0±1.7% versus 13.8±1.8%; Figure 4; P=0.15), even when adjusted for the variation in reactive hyperemia (P=0.26). There was no change in nitroglycerin-mediated vasodilation during treatment. To understand the mechanism of preserved flow-mediated vasodilation despite VEGF inhibition, membrane-associated eNOS protein level and VEGFR2 protein levels were assessed. Vandetanib application increased membrane eNOS protein levels 2-fold (P=0.04; Figure 5). No changes in VEGFR2 levels were noted.

**Discussion**

This study demonstrates that vandetanib, a VEGFR2/3, and rearranged during transfection receptor tyrosine kinase antagonist, in combination with metronomic chemotherapy, attenuates constitutive but not flow-mediated NO bioavailability in humans. Administration of this medication significantly increased mean blood pressure and decreased, in association, systemic NOx levels. These findings are bolstered by the decrease in resting conduit artery size despite the lack of change in wall shear stress and the increase in forearm vascular resistance. The mechanism of the chronic reduction in NO was tested in vitro. Vandetanib significantly reduced nitrite production and serine AKT phosphorylation in MS1 ECs. These analyses support the contention that vandetanib reduces phosphatidylinositol 3-kinase-AKT-mediated phosphorylation of eNOS activation sites and consequent attenuation in the constitutive production of NO. We also studied other potential mechanisms to explain this finding. Both eNOS and VEGFR2 total cellular and membrane fractions were evaluated in MS1 coronary ECs treated with vandetanib. The treated cells showed an increase in both total and membrane-associated eNOS but no change in either parameter for VEGFR2, providing more insight into the physiological effects of vandetanib.

**VEGF and NO**

The angiogenic and vasoactive effects of VEGF occur primarily via VEGFR2. Supporting this finding, the incidence of hypertension seen in clinical trials appears to correlate with the potency of the kinase inhibitors to block VEGFR2. VEGFR2 modulates eNOS production of NO through multiple signaling pathways. Tyrosine phosphorylation of VEGFR2 initially activates phospholipase C-γ, rapidly increasing the intracellular calcium concentration, and facilitating calmodulin-mediated eNOS disassociation from caveolin 1 and consequent NO production. Infusion of VEGF into patients induces immediate NO elaboration and hypotension. Later, VEGF activation of VEGFR2 stimulates the phosphatidylinositol 3-kinase-AKT-heat shock protein 90 pathway, which phosphorylates eNOS serine1172, a positive regulator of the enzyme.

Our findings of increased blood pressure, increased vascular resistance, decreased systemic NO production, and decreased basal arterial diameter are consistent with the predicted effects of a VEGFR2 inhibitor. Indeed, the significant reduction in systemic NOx production in this paired sample with VEGFR inhibition is greater than reported recently in another study of humans treated with VEGF antagonism. These results and our in vitro data showing that vandetanib reduces EC Akt phosphorylation and nitrite production suggest that VEGFR2 inhibition reduces constitutive eNOS-mediated NO production. Other sources of NOx are less likely to contribute to this picture, because VEGFR2 inhibition has been shown to decrease inducible NO synthase. Thus, our data are consistent with a mechanism of VEGFR2 inhibition disrupting the phosphatidylinositol 3-kinase-Akt-heat shock protein 90 phosphorylation-dependent activation of eNOS, leading to chronically reduced NO levels and hypertension, particularly in light of other work in humans.
demonstrating no change in sympathetic nervous system, renin-angiotensin system, and endothelin 1 humoral activity.\textsuperscript{15}

**Receptor Tyrosine Kinase Inhibitor Specificity and Vascular Function**

In contrast to our findings, in a study of the RTKI telatinib, there was an attenuation in vascular smooth muscle and possibly endothelial function.\textsuperscript{37} One explanation that may reconcile the differences in our observations with vandetanib versus telatinib is the variability of the effect of these RTKIs on phosphorylation of specific tyrosine moieties and cell types. The fact that telatinib and vandetanib produced different changes in blood pressure, vascular smooth muscle function, and endothelial function over a similar time course in intact humans makes clear that the description of an agent as a VEGFR2 inhibitor reveals only its gross function. A second variance may be the non-VEGFR–related effects. Telatinib inhibits platelet-derived growth factor receptor activity, which has shown to be an important mechanism by which vascular smooth muscle cells increase cytosolic calcium and may explain the attenuated response to nitroglycerin seen in this study.\textsuperscript{38,39} These results highlight the need for evaluation of each agent specifically.

**Hypertension**

Recently, several investigators have reported evidence of an association between treatment-induced hypertension and tumor responsiveness, suggesting that vascular responsiveness to therapy is a predictor of the drug’s efficacy against the cancer.\textsuperscript{40–42} This link may reflect the fact that NO mediates vascular responsiveness, as well as angiogenesis, malignant transformation, and tumorigenesis.\textsuperscript{43} These observations lead to proposals that blood pressure elevation may serve as a biomarker for efficacious VEGF signaling inhibition\textsuperscript{15} and may contribute to the cause or effect of induced hypertension. Recently, Machnik et al\textsuperscript{44} demonstrated that inhibition of VEGF receptor 3 (VEGFR3) (a described property of vandetanib) augments interstitial hypertonic volume retention, decreases eNOS expression, and increases blood pressure. Interestingly, in this experiment, inhibition of VEGF-C mediated activation of VEGFR3 was associated with a decrease in eNOS expression. Vandetanib is also an epidermal growth factor receptor antagonist and rearranged during transfection inhibitor, but blockade of these receptors has not been associated with hypertension.\textsuperscript{46} Both VEGF and eNOS contribute importantly to natriuresis,\textsuperscript{47} so the effect of renal VEGFR-2 inhibition may also mediate changes in blood pressure. Finally, the role of the metronomic therapy (methotrexate and cyclophosphamide) remains unclear. Neither is commonly associated with hypertension, but we cannot exclude any interaction. The role of these and other mechanisms need further study.

**Limitations**

This study would have been strengthened by inclusion of a control group; however, in the setting of this phase 1 trial in advanced breast cancer, randomization and the use of placebo were not possible. Whether the concomitant therapies, methotrexate and cyclophosphamide, contributed to vascular function remains an open question, but they have not demonstrated this tendency in past studies of subjects with rheumatological disease.\textsuperscript{48–52} Diet is an important contributor to levels of NOx. A change in diet may have participated in changing the systemic level of NOx; however, we believe the trend noted between increasing blood pressure and decreasing NOx suggests that these 2 changes were related.

**Perspectives**

Treatment with the VEGFR2 RTKI vandetanib in combination with metronomic chemotherapy increased blood pressure, decreased constitutive NO production, and decreased conduit artery resting diameter. In vitro, vandetanib decreased Akt phosphorylation and NO production, yet increased nitrite levels in our subjects. These findings are similar to other RTKIs. We cannot exclude the possibility of an increase in the production of an endogenous vasoconstrictor; however, in patients treated with the VEGFR2 inhibitor sorafenib, despite a mean increase in blood pressure of 13 mm Hg, there were no changes in catecholamines, endothelin I, urotensin II, renin, and aldosterone levels.\textsuperscript{15} Additional agent-specific mechanisms are likely present and require further study to elucidate.

Additional mechanisms of hypertension need to be considered. Vascular rarefaction has been demonstrated in response to VEGF antagonism\textsuperscript{37} and may contribute to the cause or effects of induced hypertension. Recently, Machnik et al\textsuperscript{44} demonstrated that inhibition of VEGF receptor 3 (VEGFR3) (a described property of vandetanib) augments interstitial hypertonic volume retention, decreases eNOS expression, and increases blood pressure. Interestingly, in this experiment, inhibition of VEGF-C mediated activation of VEGFR3 was associated with a decrease in eNOS expression. Vandetanib is also an epidermal growth factor receptor antagonist and rearranged during transfection inhibitor, but blockade of these receptors has not been associated with hypertension.\textsuperscript{46} Both VEGF and eNOS contribute importantly to natriuresis,\textsuperscript{47} so the effect of renal VEGFR-2 inhibition may also mediate changes in blood pressure. Finally, the role of the metronomic therapy (methotrexate and cyclophosphamide) remains unclear. Neither is commonly associated with hypertension, but we cannot exclude any interaction. The role of these and other mechanisms need further study.

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**Disclosures**

None.

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Contrary Effects of the Receptor Tyrosine Kinase Inhibitor Vandetanib on Constitutive and Flow-Stimulated Nitric Oxide Elaboration in Humans

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