The Tyrosine Kinase Inhibitor Cediranib Blocks Ligand-Induced Vascular Endothelial Growth Factor Receptor-3 Activity and Lymphangiogenesis

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Abstract

Solid tumors express a range of factors required to sustain their growth and promote their dissemination. Among these are vascular endothelial growth factor-A (VEGF-A), the key angiogenic stimulant, and VEGF-C, a primary mediator of lymphangiogenesis. Small molecule tyrosine kinase inhibitors offer the potential to inhibit more than one kinase and impede tumor growth by multiple mechanisms. However, their potency toward individual targets can vary. Cediranib (RECENTIN; AZD2171) is an inhibitor of VEGF signaling that has been shown in experimental models to prevent VEGF-A–induced angiogenesis and primary tumor growth, yet the effects of cediranib on VEGF receptor (VEGFR)-3–mediated endothelial cell function and lymphangiogenesis are unknown. To better understand the activity of cediranib against VEGFR-3 and its associated signaling events compared with its activity against VEGFR-2, we used the receptor-specific ligands VEGF-E and VEGF-C156S. In human endothelial cells, cediranib inhibited VEGF-E–induced phosphorylation of VEGFR-2 and VEGF-C156S–induced phosphorylation of VEGFR-3 at concentrations of ≤1nmol/L and inhibited activation of downstream signaling molecules. Additionally, cediranib blocked VEGF-C156S–induced and VEGF-E–induced proliferation, survival, and migration of lymphatic and blood vascular endothelial cells. *In vivo*, cediranib (6 mg/kg/d) prevented angiogenesis and lymphangiogenesis induced by VEGF-E–expressing and VEGF-C156S–expressing adenoviruses, respectively. Cediranib (6 mg/kg/day) also blocked angiogenesis and lymphangiogenesis induced by adenoviruses expressing VEGF-A or VEGF-C and compromised the blood and lymphatic vasculatures of VEGF-C–expressing tumors. Cediranib may, therefore, be an effective means of preventing tumor progression, not only by inhibiting VEGFR-2 activity and angiogenesis, but also by concomitantly inhibiting VEGFR-3 activity and lymphangiogenesis. [Cancer Res 2008;68(12):4754–62]

Introduction

Angiogenesis and neovascularization are important mechanisms that tumors use to promote their growth and metastasis (1). Increased tumor growth is dependent on the oxygen and nutrients provided by tumor blood vessels, which also facilitate the dissemination of malignant cells to distal sites. Metastatic cancer spread is further enhanced by an increase in lymphatic vessel growth or lymphangiogenesis in and around the tumor (2, 3). For many types of solid tumors, the lymphatic system is the primary conduit for initial metastasis. Malignant cells transported via lymphatic capillaries and vessels spread to the regional lymph nodes and are eventually routed into the blood circulation where they metastasize to the lungs and other distant organs.

The vascular endothelial growth factor (VEGF) family of ligands and receptors are potent inducers of angiogenesis and lymphangiogenesis. VEGF-A binds and activates VEGF receptor-1 (VEGFR-1; also called Flt-1) and VEGFR-2 (also called KDR) expressed on endothelial cells and initiates a signal transduction cascade affecting numerous processes required for the formation of blood capillaries (4). These include endothelial cell proliferation, survival, migration, tube formation, recruitment of endothelial cell precursors, pericyte and mural cell interaction, vascular permeability, and altered integrin and protease expression. Lymphangiogenesis is primarily driven by VEGF-C and VEGF-D, which activate VEGFR-3 (also called Flt-4) and induce a signaling cascade similar to that induced by VEGF-A acting on VEGFR-2 (5, 6). VEGFR-3 is expressed early in the development on the blood vessel endothelium and is required for remodeling and maturation of the primary vascular plexus. Later, VEGFR-3 becomes confined to expression on some fenestrated blood vessels, but is primarily expressed by lymphatic endothelial cells (LEC) and is required for development of the lymphatic system (7, 8). In the adult, VEGFR expression is largely reduced on the mature blood and lymphatic vasculatures, with expression remaining high in only certain vascular beds (9, 10). These remain dependent on VEGF signaling for survival, although they exhibit remarkable plasticity when signaling is blocked and then resumed (10). During physiologic and pathologic conditions, including cancer, the VEGFRs are up-regulated and VEGFR-3 cannot only be found on tumor-associated lymphatics, but also on angiogenic tumor blood vessels (11, 12).

Given that expression of VEGFRs is reduced in the adult on quiescent vessels, but up-regulated during pathologic angiogenesis and lymphangiogenesis, these receptors and their ligands are particularly attractive targets for therapeutic intervention. Approaches to inhibit VEGF signaling include monoclonal antibodies that target VEGF ligands or receptors (13), soluble receptors that sequester ligands (14), and small molecule inhibitors that compete for the ATP-binding site within the receptor kinase domain (15). Whereas antibodies and receptor constructs are highly specific, small molecule inhibitors can inhibit more than one

Note: T. Holopainen and M. Wirzenius contributed equally to this work.

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kinase particularly within a structurally related class. Considering the complexity of the angiogenic and lymphangiogenic processes, targeting more than one VEGFR is likely required for successful inhibition of tumor blood and lymphatic vessel growth.

Cediranib (RECENTIN; AZD2171) is an indole-ether quinazoline that is a potent VEGF signaling inhibitor, with additional activity against c-Kit that was previously shown to prevent both physiologic and pathologic angiogenesis *in vivo* (16). Cediranib inhibited the growth of a number of different human tumor xenografts (16, 17) and reduced tumor growth in a mouse model of spontaneous intestinal cancer (18). The ability of cediranib to inhibit tumor growth was associated with a decrease in the tumor blood vasculature preceded by a reduction of VEGFR-2 phosphorylation evident within 28 hours of treatment (16, 19).

Early clinical studies have shown encouraging antitumor activity in patients with a broad range of solid tumors, as well as time-dependent and dose-dependent changes in pharmacodynamic markers (20). Results from a recently completed clinical trial have shown that daily administration of cediranib to glioblastoma patients resulted in a rapid and prolonged normalization of the tumor vasculature that subsequently led to a reduction in tumor-associated edema (21). Cediranib is presently in phase II/phase III clinical development.

VEGFR-2 is a key target for cediranib inhibition of VEGF signaling, and cediranib has been shown by recombinant kinase assays to be active against the other VEGFRs (16). For these studies, we wished to compare the ability of cediranib to inhibit VEGFR-2–driven signaling and angiogenesis with its effects on VEGFR-3–specific signaling and lymphangiogenesis. For this purpose, we used two VEGF family ligands with unique binding properties: the viral homologue of VEGF-A, called VEGF-E (22), which activates only VEGFR-2, and a mutant form of VEGF-C, called VEGF-C156S, which preferentially activates VEGFR-3 (23). Cediranib inhibited VEGF-E–induced VEGFR-2 activity and VEGF-C156S–induced VEGFR-3 activity within the same concentration range (1 nmol/L) in human endothelial cells, and blocked both ligand-driven and tumor cell–driven angiogenesis and lymphangiogenesis *in vivo*.

Materials and Methods

Reagents and cells. For *in vitro* experiments, cediranib (AstraZeneca Pharmaceuticals) was resuspended in DMSO and diluted in cell media for the relevant assays. For *in vivo* experiments, cediranib was dissolved in 1% aqueous polysorbate 80 at 0.3 and 0.6 mg/mL. Human dermal microvascular endothelial cells (HDMVEC) were maintained in endothelial cell media (PromoCell) according to the supplier's instructions and used at passages 3 to 8. The NCI-H460-LNM35 (LNM35) cell line is a human non–small cell lung adenocarcinoma derived from NCI-H460-N15 cells (24). LNM35 cells stably expressing the enhanced green fluorescent protein (EGFP) were established and maintained as described (24, 25). VEGF-C and the VEGF-C156S mutant were prepared as described (26). The expression construct used to produce VEGF-C156S was identical to that used to produce wild-type VEGF-C except for the G to C mutation at nucleotide 818 of the VEGF-C coding sequence (Genbank accession no. X94216). VEGF-E was prepared as described (27). The following antibodies were used: VEGFR-2 and VEGFR-3 (Santa Cruz Biotechnology); phosphorylated tyrosine (Upstate Biotechnology); phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), ERK1/2, phosphorylated Akt, Akt, phosphorylated cAMP-responsive element binding protein (CREB), and CREB (Cell Signaling Technology); Prox-1 and activated caspase-3 (R&D Systems); platelet/endothelial cell adhesion molecule 1 (PECAM-1; BD Biosciences PharMingen); and Alexa Fluor 488 and 594 secondary antibodies (Invitrogen/Molecular Probes). Rabbit serum against the lymphatic hyaluronan receptor LYVE-1 was prepared as described (28). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma-Aldrich.

Immunoprecipitation and Western analysis. For receptor phosphorylation studies, HDMVECs were grown to confluency on fibronectin-coated (Sigma-Aldrich) dishes. The cells were serum-starved overnight, the medium was replaced with fresh serum-free medium, DMSO or cediranib was added, and incubation was carried out for 15 min, after which VEGF-C156S, VEGF-E, or bovine serum albumin (BSA; control protein) was added (500 ng/mL) and incubation continued for 10 min. The cells were rinsed with cold PBS and then lysed in PLCLB buffer [50 mmol/L HEPES (pH 7.5), 1% Triton X-100, 5% glycerol, 1 mmol/L EGTA, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 100 mmol/L NaF] containing protease inhibitors (0.5 mmol/L phenylmethylsulfonyl fluoride, 15 μg/mL aprotinin, 10 μg/mL leupeptin, 2 mmol/L Na_3VO_4). Protein concentrations were measured by Bradford assay, and equal amounts of protein immunoprecipitated with VEGFR-2 or VEGFR-3 antibodies in the presence of 0.5% BSA, 0.02% Tween 20 then pulled down with protein A–sepharose (Amersham Biosciences). Sepharose beads were washed with PLCLB containing Na_3VO_4 before resuspension in Laemmli's buffer. The samples were separated by 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience GmbH). Phosphorylated receptors were detected using the phosphorylated tyrosine antibody, a biotinylated secondary antibody (DakoCytomation), and streptavidin–biotinylated horseradish peroxidase conjugate (Amersham Biosciences). Signals were visualized by chemoluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology). Membranes were stripped and reprobed for VEGFR-2 or VEGFR-3. For detection of downstream signaling molecules, confluent HDMVEC cultures were serum-starved and stimulated as above. Lysates were prepared, and equal amounts of protein were separated by 12% SDS-PAGE before transferring to nitrocellulose. Detection was carried out using phosphorylated ERK1/2, phosphorylated Akt, and phosphorylated CREB antibodies. Membranes were stripped and reprobed with the ERK1/2, Akt, or CREB antibodies.

Endothelial cell proliferation. The separation of LECs and blood endothelial cells (BEC) was performed as described (29). Either 3,000 LECs or 3,000 BECs were seeded per well in fibronectin-treated 96-well plates and allowed to attach overnight. The medium was replaced with serum-free medium containing cediranib or DMSO with 500 ng/mL VEGF-C156S or VEGF-E. The cells were maintained at 37°C for 8 d. MTT was added (625 μg/mL), and incubation continued for 3 h. The medium was removed, and the cells lysed with DMSO. MTT incorporation was measured with a spectrophotometer at 540 nm. Data were collected from eight replicates.

Endothelial cell apoptosis. LECs and BECs were separated, and 60,000 cells were seeded onto fibronectin-coated glass coverslips in 24-well plates and allowed to attach overnight before serum starvation for 24 h. The serum-free medium was replaced and DMSO or cediranib was added, and the cells were incubated for 15 min. VEGF-E or VEGF-C156S (500 ng/mL) was added, and incubation continued for 18 h. The cells were fixed with 4% PFA, washed with PBS, and permeabilized with 0.1% Triton X-100. Coverslips were blocked with 2% BSA and 0.1% Triton X-100 in PBS, then incubated with Prox-1 and activated caspase-3 antibodies diluted in blocking buffer. The coverslips were rinsed with PBS, incubated with secondary antibodies, and then rinsed again before mounting on glass slides with Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). Images were collected on a Zeiss LSM 510 Meta confocal microscope with a 40× plan-neofluar objective. Prox-1–positive staining confirmed LEC identity, whereas Prox-1–negative staining confirmed BEC identity. Caspase-3 was used to distinguish apoptotic cells from non-apoptotic cells. Relative apoptosis was calculated as the percentage of caspase-3–positive cells in the test samples divided by the percentage of caspase-3–positive cells in the nonstimulated control samples.

Endothelial cell migration. Assessment of endothelial cell migratory activity was performed as described (30). Separated LECs and BECs were suspended in serum-free media and seeded in the top chamber of a cell culture insert (BD Biosciences Discovery Labware) with DMSO or cediranib. The insert was placed in a 24-well plate containing serum-free medium with control protein, VEGF-C156S or VEGF-E (500 ng/mL); the cells were

incubated for 24 h then fixed with ice-cold methanol. Nonmigrated cells were removed, and migrated cells were stained with Giemsa (Merck). Migratory activity was calculated by dividing the number of responsive cells in the test conditions by the number of cells migrated in the control samples.

Generation of recombinant adenoviruses. Adenoviruses encoding VEGF-C, VEGF-C156S, VEGF-A, VEGF-E, and nuclear targeted β-galactosidase (*LacZ*) were constructed and produced as described (31–35). Protein expression by the adenoviruses was confirmed by *in vitro* analysis as published (36). Briefly, adenoviruses were used to infect HeLa cell cultures that were then metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (Redivue ProMix, Amersham Biosciences) in methionine-free and cysteine-free media. Supernatants were collected from the transduced cells and analyzed by SDS-PAGE.

Inhibition of angiogenesis and lymphangiogenesis. All animal experiments were approved by the Provincial State Office of Southern Finland and carried out in accordance with institutional guidelines. For the induction of angiogenesis and lymphangiogenesis, 6-week-old to 8-week-old male NMRI nu/nu mice (Taconic) were anesthetized with Ketalar (Pfizer) and Rompun (Bayer) before i.d. injection of 5×10^8 plaque-forming units (pfu) of recombinant adenoviruses into each ear. Eight mice were used for each virus type. The mice were further subdivided into groups of four and given cediranib (6 mg/kg/d) or vehicle (1% polysorbate) by p.o. gavage daily, starting from the day of adenovirus injection. One week after injection, the mice were sacrificed and the ears were prepared for whole mount staining and analysis performed as described (37). Lymphatic and blood vessels were detected using the LYVE-1 and PECAM-1 antibodies, respectively, and the appropriate fluorochrome-conjugated secondary antibody. The ears were mounted onto glass slides using Vectashield and analyzed on a confocal microscope with a 10× fluar NA 0.5 objective. Three-dimensional projections were digitally constructed from confocal z-stacks. Quantification of the area covered by lymphatic or blood vessels in the skin was performed as published (38). A total of eight ears were analyzed per data set, and LYVE-1 and PECAM-1 staining was quantified using the ImageJ program (Media Cybernetics). All statistical analyses were performed using the unpaired two-tailed Student's *t* test. A P value of <0.05 was considered statistically significant. For tumor cell–induced angiogenesis and lymphangiogenesis, LNM35/EGFP cells were washed and suspended in PBS at 5×10^4 cells/μL. Female NMRI nu/nu mice were anesthetized, and 1×10^6 cells (20 μL) were i.d. injected into each ear. The mice were separated into three groups with six mice per group and treated p.o. daily with cediranib (3 or 6 mg/kg/d) or vehicle starting from the day of injection. The mice were humanely sacrificed 2 wk later, their ears were prepared for whole mount staining, with blood and lymphatic vessels detected, and analysis was performed as described above. Six tumors were evaluated for each data set, with images taken from at least three regions.

Changes in vascular permeability. Male NMRI nu/nu mice (6–8 wk old) were divided into two groups, assigned either cediranib (6 mg/kg/d) or control vehicle. The mice were weighed and given the assigned treatment by p.o. gavage. The two groups were each subdivided into three more

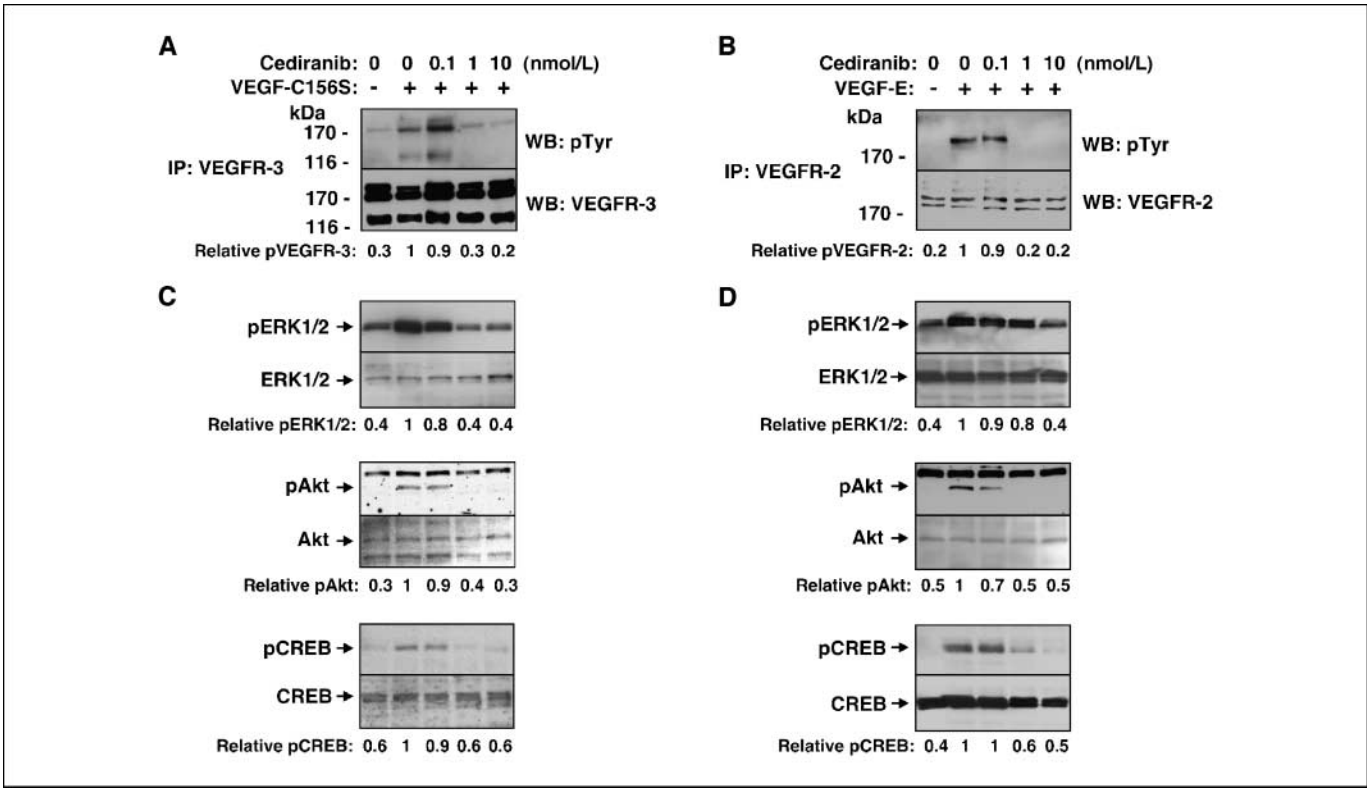

Figure 1. Cediranib inhibits specific activation of VEGFR-2 and VEGFR-3 and associated downstream signals in human endothelial cells. Confluent cultures of HDMVECs were placed in serum-free and growth factor–free endothelial cell media overnight before pretreatment with cediranib or an equivalent volume of DMSO for the nontreated controls, and then stimulation with 500 ng/mL VEGF-C156S or VEGF-E. Lysates prepared from the cells were subjected to immunoprecipitation (*IP*) with VEGFR-3–specific or VEGFR-2–specific antibodies before separation by 7.5% SDS-PAGE (*A* and *B*), or the lysates were separated directly by 12% SDS-PAGE (*C* and *D*). The proteins were transferred to nitrocellulose and detected by Western blotting (*WB*) using phospho-specific antibodies (*top*) followed by detection with protein-specific antibodies (*bottom*). *A,* the 180-kDa and 125-kDa isoforms of VEGFR-3 are phosphorylated on tyrosine residues when HDMVECs are incubated with VEGF-C156S. Increased phosphorylation of both isoforms is prevented when the cells are pretreated with 1 or 10 nmol/L of cediranib. *B,* stimulation of HDMVECS with VEGF-E results in tyrosine phosphorylation of VEGFR-2, which is ~220 kDa. Phosphorylation is inhibited with a pretreatment of 1 or 10 nmol/L of cediranib. *C,* stimulation of HDMVECs with VEGF-C156S results in increased phosphorylation of ERK1/2, Akt, and CREB. This activity is inhibited when the cells are pretreated with at least 1 nmol/L cediranib. *D,* increased phosphorylation of ERK1/2, Akt, and CREB is observed when HDMVECs are stimulated with VEGF-E. The increased activation/phosphorylation of all three signaling molecules is reduced when the cells are pretreated with a concentration of ≥1 nmol/L of cediranib.

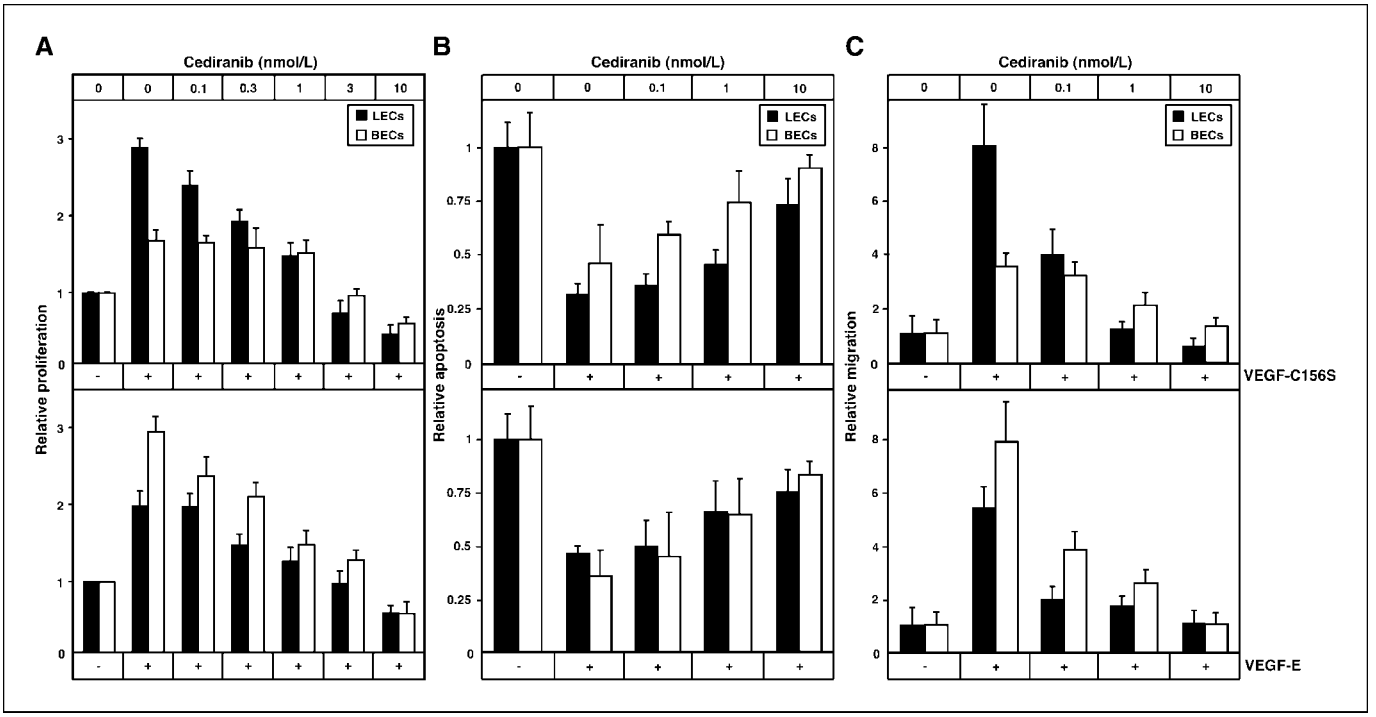

Figure 2. Cediranib inhibits ligand-induced proliferation, survival, and migration of LECs and BECs. LECs and BECs were separated from HDMVEC cultures and maintained independently before their use in each assay. *A*, quantification of MTT incorporated by LECs (*black columns*) or BECs (*white columns*) incubated in serum-free conditions in the absence or presence of 500 ng/mL VEGF-C156S or VEGF-E and DMSO or 0.1 to 10 nmol/L of cediranib. *Columns,* mean value of eight replicates normalized to the untreated controls; *bars,* SE. Cediranib inhibited the ligand-induced growth of both LECs and BECs. *B*, quantification of activated caspase-3 in LECs or BECs maintained in serum-free conditions with the addition of VEGF-C156S or VEGF-E, along with DMSO or 0.1, 1, or 10 nmol/L cediranib. The data represent the mean value of five replicates, in which positive staining for cleaved caspase-3 indicated cells undergoing apoptosis. For each sample, the ratio of caspase-3–positive cells to the total number of cells was calculated. These values were then normalized to the value calculated for the nonstimulated control cells and indicated as relative apoptosis. On average, ~73% LECs and 45% BECs were apoptotic in serum-free and growth factor–free conditions. In a dose-dependent manner, cediranib was able to abrogate the ability of VEGF-C156S and VEGF-E to prevent apoptosis induced by serum deprivation. *C,* quantification of LEC and BEC migratory activity induced by VEGF-C156S or VEGF-E and treated with DMSO, 0.1, 1, or 10 nmol/L cediranib. The number of migrated cells from test samples was divided by the number of migrated cells in the nonstimulated controls, and these values are indicated as relative migration. *Columns,* mean values from four independent experiments. Cediranib reduced ligand-stimulated migration of LECs and BECs.

groups (n = 4) and assigned AdVEGF-A, AdVEGF-E, or AdLacZ virus. The mice were anesthetized, and each ear was injected i.d. with 5×10^8 pfu of recombinant adenoviruses. Administration of cediranib or vehicle was continued daily for 7 d. After this period, the mice were anesthetized and 3% Evans Blue (1 mL/g) was injected via the lateral tail vein 10 min before sacrifice. The ears were dissected, weighed, immersed in formamide, and incubated overnight at 55°C. Evans Blue leakage between samples was calculated by measuring the absorbance of the formamide at 620 nm on a spectrophotometer. These measurements were divided by the weight of the corresponding ear, and the relative leakage was calculated by normalizing to the control vehicle AdLacZ value. Eight ears were evaluated for each data set.

Results

Cediranib inhibits specific activation of VEGFR-2 and VEGFR-3 and associated downstream signaling molecules in human endothelial cells. To examine the effects of cediranib on ligand-induced VEGFR activity, we used primary HDMVECs, which are a mixed population of BECs and LECs. VEGFR-2 is expressed on both cell types, and while VEGFR-3 is expressed predominantly on LECs, it is also expressed to some extent on BECs (39, 40). VEGF-C156S induced phosphorylation of the 180-kDa full-length and 125-kDa processed forms of VEGFR-3, with 1 nmol/L of cediranib sufficient to reduce induced phosphorylation of both isoforms (Fig. 1*A*). Similarly, VEGF-E induced the phosphorylation of VEGFR-2, and cediranib blocked this activation at 1 nmol/L (Fig. 1*B*). After ligand-induced VEGFR autophosphorylation, a number of signaling events take place. These include activation of the mitogen-activated protein kinase (MAPK) and ERK pathway, which mediates cell proliferation, and the phosphotidylinositol 3-kinase pathway, which is linked to proliferation, but also controls cell survival by activation of protein kinase B (Akt). VEGF-C156S or VEGF-E stimulation of HDMVECs resulted in increased phosphorylation of p42/p44-MAPK (ERK1/2) and Akt, as well the CREB protein, a transcription factor linked to both cell proliferation and survival (Fig. 1*C* and *D*). Whereas 1 nmol/L of cediranib was sufficient to block Akt and CREB phosphorylation, higher concentrations were required to interfere with ERK1/2 activation stimulated by VEGFR-2 signaling (Fig. 1*C* and *D*).

Cediranib inhibits VEGFR-2 and VEGFR-3 induction of proliferation, survival, and migration of BECs and LECs. For comparative analyses of cediranib effects on LEC and BEC functional activity, proliferation assays were performed on separated cultures. Stimulation with VEGF-C156S resulted in a significant increase in LEC proliferation, along with a slight increase in BEC growth (Fig. 2*A, top*). A dose-dependent decrease in VEGF-C156S–induced proliferation was observed upon addition of cediranib with 3 nmol/L, sufficient to bring the induced proliferation to background level (Fig. 2*A, top*). Stimulation with VEGF-E resulted in a strong induction of BEC proliferation and a

smaller induction of LEC proliferation (Fig. 2*A, bottom*). However, the addition of cediranib resulted in a dose-dependent decrease of VEGF-E–induced proliferation with 3 nmol/L, sufficient to bring both LEC and BEC growth down near to background levels (Fig. 2*A, bottom*). LECs and BECs maintained in serum and growth factor–free conditions undergo apoptosis; however, the apoptotic process can be prevented with the addition of VEGF-C156S or VEGF-E as measured by the presence of activated caspase-3 (Fig. 2*B*). The addition of cediranib reduced the ability of the ligands to rescue both BECs and LECs from serum deprivation-induced apoptosis at those concentrations that also prevented activation of Akt, which is known to provide survival signaling (Figs. 1*C* and *D* and 2*B*). As a further measure of the effects of cediranib on LEC and BEC function, we also examined its ability to inhibit VEGFR-2–mediated or VEGFR-3–mediated cell migration. To measure this activity, LECs or BECs suspended in serum and growth factor–free media were placed in the top chamber of a modified Boyden chamber along with control vehicle or cediranib, whereas serum-free media was placed in the lower chamber along with control protein or VEGF-C156S or VEGF-E. VEGF-C156S and VEGF-E elicited a strong migratory response from LECs, whereas BECs responded more to VEGF-E stimulation (Fig. 2*C*). Interestingly, 0.1 nmol/L of cediranib was sufficient to reduce VEGF-C156S–induced and VEGF-E–induced LEC migration, as well as VEGF-E–induced BEC migration by ~50% or more (Fig. 2*C*).

Cediranib blocks specific induction of angiogenesis and lymphangiogenesis. To assess the effects of cediranib on ligand-induced lymphangiogenesis and angiogenesis *in vivo*, we used recombinant adenoviruses expressing VEGF-C, VEGF-C156S, VEGF-A, VEGF-E, as well as the control LacZ. The adenoviruses were i.d. injected into each ear of the mice, and the mice were treated for 7 days with cediranib (6 mg/kg) or control vehicle. Adenoviruses expressing VEGF-C or VEGF-C156S were powerful inducers of lymphangiogenesis, as noted from the disorganized architecture of the lymphatic vasculature and increased lymphatic sprouting observed by staining for LYVE-1 (Fig. 3*A*). Similarly, the VEGF-A expressing adenovirus induced lymphangiogenesis as noted by the presence of lymphatic sprouts (Fig. 3*A*). Whereas the adenovirus expressing VEGF-E did not induce obvious lymphatic sprouting, lymphatic vessels seemed slightly enlarged in the AdVEGF-E–treated ears compared with those of the AdLacZ control ears (Fig. 3*A*) and as noted by evaluation of the LYVE-1–positive vessel area (Fig. 3*B*). However, cediranib treatment effectively prevented lymphangiogenesis induced by VEGF-C, VEGF-C156S, and VEGF-A and lymphatic hyperplasia induced by VEGF-E (Fig. 3*A* and *B*). In the blood vasculature, adenoviruses expressing VEGF-C156S or the LacZ control had little effect on vessel number or size, whereas adenoviruses expressing VEGF-A and VEGF-E induced extensive remodeling accompanied by an increase in the number of blood capillaries, as shown by PECAM-1 staining (Fig. 3*C*). The adenovirus expressing VEGF-C also induced a slight increase in the blood vasculature (Fig. 3*C*), which was likely due to the ability of processed VEGF-C to bind and activate VEGFR-2 and possibly the expression of VEGFR-3 on angiogenic blood vessels. The blood vessels of cediranib-treated animals, however, seemed normal and were comparable with those of the controls, indicating that cediranib efficiently blocked VEGFR-mediated angiogenesis (Fig. 3*C* and *D*).

Cediranib affects vascular permeability. VEGF ligands known to activate VEGFR-1 and VEGFR-2 also mediate vessel permeability; thus, we examined the effects of cediranib on VEGF-A–induced and VEGF-E–induced vascular leakage. For this assessment, we injected the VEGF-A, VEGF-E, or LacZ adenoviruses in the ears of nude mice and treated the mice with cediranib (6 mg/kg) or control vehicle for 7 days. Changes in vessel permeability were measured using a dye extravasation assay with Evans Blue dye injected i.v. Compared with AdLacZ control-treated animals, a change in blood vessel permeability was most notable with AdVEGF-A, whereas AdVEGF-E had only a small effect (Fig. 4). However, cediranib efficiently inhibited the vascular leakage induced by both VEGF-A and VEGF-E (Fig. 4). Whereas high levels of VEGF-C can also induce blood vessel permeability (36), this is believed to be due to the ability of processed VEGF-C to bind and activate VEGFR-2, as VEGF-C156S has previously been shown to have only a very minimal effect on vessel leakage (33).

Cediranib prevents tumor cell–induced angiogenesis and lymphangiogenesis. Whereas most tumor types express VEGF-A to induce the blood vessel growth required to supply the nutrients and oxygen needed to sustain their increase in mass, many tumors also express VEGF-C to induce the growth of lymphatic vessels used for dissemination. An example of a VEGF-C–expressing tumor cell line is LNM35, which is a highly metastatic subclone of a human non–small cell lung adenocarcinoma (41). LNM35 cells stably expressing EGFP were implanted s.c. into both ears of nude mice. The mice were p.o. treated with vehicle or cediranib (3 or 6 mg/kg), with treatment carried out once daily for 2 weeks starting from the day of tumor cell implantation. Examination of the tumor vasculatures showed that, whereas tumors from control mice displayed a very dense network of blood capillaries as observed by PECAM-1 staining, tumor blood vessels of mice treated with cediranib at 3 and 6 mg/kg/day were far fewer (Fig. 5*B*). As observed by LYVE-1 staining, control tumors displayed numerous lymphatic vessels/capillaries (Fig. 5*B*). In contrast, there were fewer lymphatic vessels associated with the tumors of mice treated with cediranib (Fig. 5*B*). Quantification of the blood and lymphatic vessels associated with these tumors showed that 3 mg/kg/day of cediranib dramatically reduced the number of both vessel types (Fig. 5*C* and *D*). Although a higher concentration of cediranib did not further reduce the number of tumor-associated blood vessels compared with the lower dose (Fig. 5*C*), tumor-associated lymphatic vessels were further compromised with the 6 mg/kg/day dose of cediranib compared with the 3 mg/kg/day treatment (Fig. 5*D*; $P = 0.039$). In general, cediranib prevented the growth of the implanted tumors (Fig. 5*A, top*). The effect on tumor growth *in vivo*, however, was not likely due to a direct effect of cediranib on tumor cell proliferation, as even 5 μmol/L of cediranib did not inhibit proliferation of LMN35 cells *in vitro* (data not shown). In comparison to concentrations inhibiting LEC and BEC proliferation *in vitro*, the concentration of cediranib required to prevent LNM35 cell proliferation was much greater (1 nmol/L compared with >5 μmol/L).

Discussion

Cediranib was previously shown to be an effective inhibitor of VEGF-induced signaling and angiogenesis, and by enzyme assays, cediranib was also found to have activity against VEGFR-1 and VEGFR-3 (16). We now show that cediranib blocks VEGFR-3–mediated endothelial cell functions and lymphangiogenesis at similar doses that inhibit VEGFR-2 activity and angiogenesis. In many types of cancer, expression of the VEGFR-3 ligands VEGF-C and VEGF-D is correlated with the occurrence of lymph node

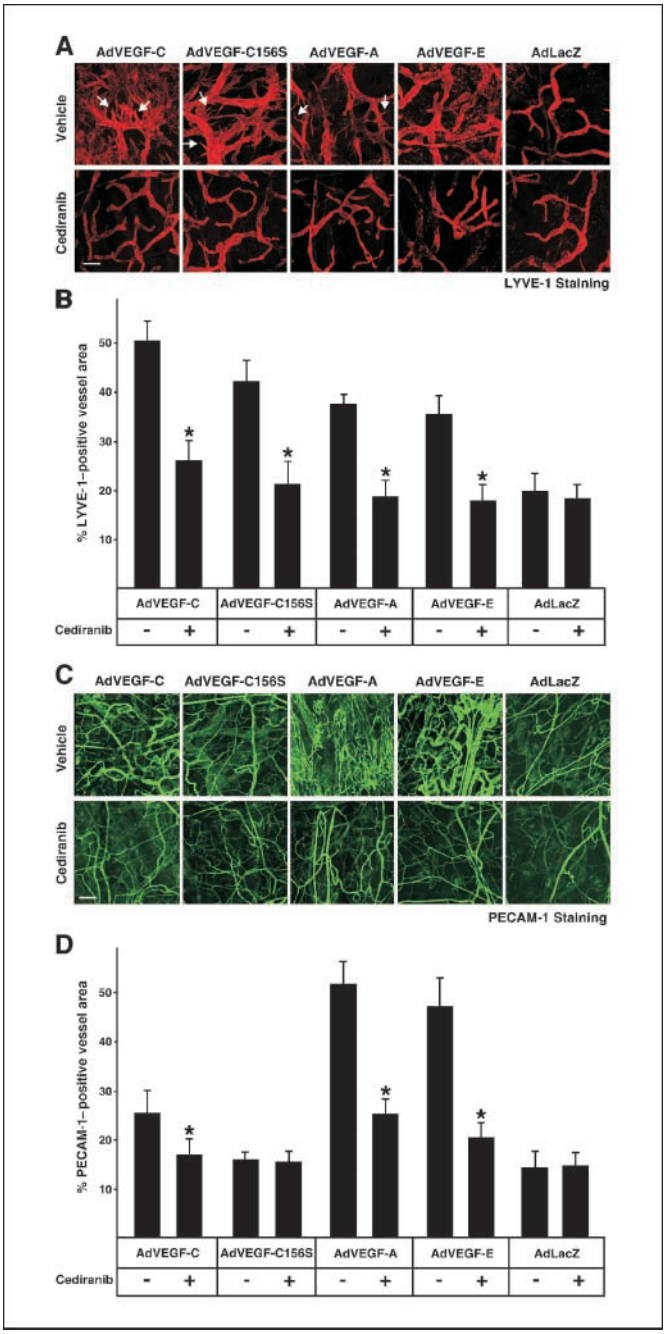

Figure 3. Cediranib prevents ligand-induced lymphangiogenesis and angiogenesis. *A*, whole mount staining for the lymphatic endothelium marker LYVE-1 of indicated adenovirus vector-transduced ears. AdVEGF-C, AdVEGF-C156S, and AdVEGF-A transduction induced lymphatic sprouting, whereas AdVEGF-E transduction induced lymphatic hyperplasia compared with the AdLacZ-transduced ears. Treatment with cediranib (6 mg/kg/d) reduced the sprouting and hyperplasia of the lymphatic vessels induced by AdVEGF-C, AdVEGF-C156S, AdVEGF-A, and AdVEGF-E. *Arrows,* sprouts. The white line represents 200 μm. *B,* quantification of LYVE-1–positive vessel density. The asterisks indicate statistically significant ($P \leq 0.05$) decreased lymphatic vessel density area. *C,* whole mount staining for the blood vessel endothelium marker PECAM-1 of indicated adenovirus vector-transduced ears. AdVEGF-A and AdVEGF-E transduction induced a strong angiogenic response, whereas AdVEGF-C transduction resulted in a weaker response. AdVEGF-C156S transduction, however, had no angiogenic effect compared with the control AdLacZ-transduced ears. Treatment with cediranib (6 mg/kg/d) reduced AdVEGF-C–induced and AdVEGF-E–induced angiogenesis. The white line represents 200 μm. *D*, quantification of PECAM-1–positive vessel density. The asterisks indicate statistically significant ($P \leq 0.05$) decreased vessel density area.

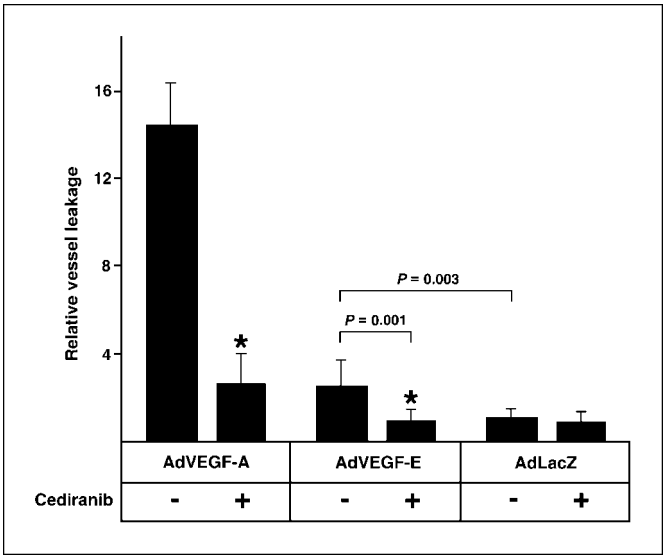

Figure 4. Cediranib inhibits ligand-induced changes in vessel permeability. Extravasation of Evans Blue was quantified from AdVEGF-A–, AdVEGF-E–, and AdLacZ–transduced ears 10 min after i.v. injection of the dye. The absorbance of Evans Blue leakage into formamide was measured on a spectrophotometer at 620 nm, and these numbers were divided by the weight of the corresponding ear. The resulting values were normalized to the nontreated AdLacZ value and indicated as relative vessel leakage. Whereas dye leakage was greatest in the AdVEGF-A–transduced ears, some leakage was also apparent with AdVEGF-E. Vessel leakage was inhibited with cediranib treatment (6 mg/kg/d).

metastasis, an indication of disease progression and prognosis for reduced survival (42). In addition, the formation of lymph node metastasis can be promoted by VEGF-C and inhibited with a soluble form of VEGFR-3 or a monoclonal antibody targeting the receptor (41, 43, 44). VEGFR-3 may also have a role in pathologic angiogenesis, such as tumor blood vessel formation, and the use of a VEGFR-3–specific antibody has been shown to reduce primary tumor growth (45). Inhibition of VEGFR-3 activity is therefore likely to be therapeutically beneficial.

Although VEGF-C and VEGF-D bind and regulate VEGFR-3 activity, the fully processed forms of these two ligands also bind and activate VEGFR-2 and induce its associated responses (46, 47). Therefore, we used the specific VEGFR-2 ligand VEGF-E and the VEGFR-3 ligand VEGF-C156S to differentiate the effects of cediranib on VEGFR-2 and VEGFR-3 signaling *in vitro* and *in vivo*. At 1 nmol/L, cediranib inhibited VEGF-C156S–induced phosphorylation of VEGFR-3 and VEGF-E–induced phosphorylation of VEGFR-2 in primary human endothelial cells. This was accompanied by reduced activation of downstream signaling molecules ERK1/2, Akt, and CREB. Additionally, cellular functions associated with VEGFR activation, including proliferation, survival, and migration, were all compromised by cediranib. Proliferation of LECs and BECs induced by either VEGF-C156S or VEGF-E was reduced with low levels of cediranib, whereas higher concentrations attenuated the ability of VEGF-C156S and VEGF-E to prevent LECs and BECs from undergoing apoptosis induced by serum deprivation. Interestingly, cellular migration seemed to be the most sensitive to cediranib treatment, with VEGF-C156S–induced and VEGF-E–induced LEC migration and VEGF-E–induced BEC migration compromised at 0.1 nmol/L, although there was only slight inhibition of ligand-induced receptor phosphorylation at this concentration. The tyrosine residues of

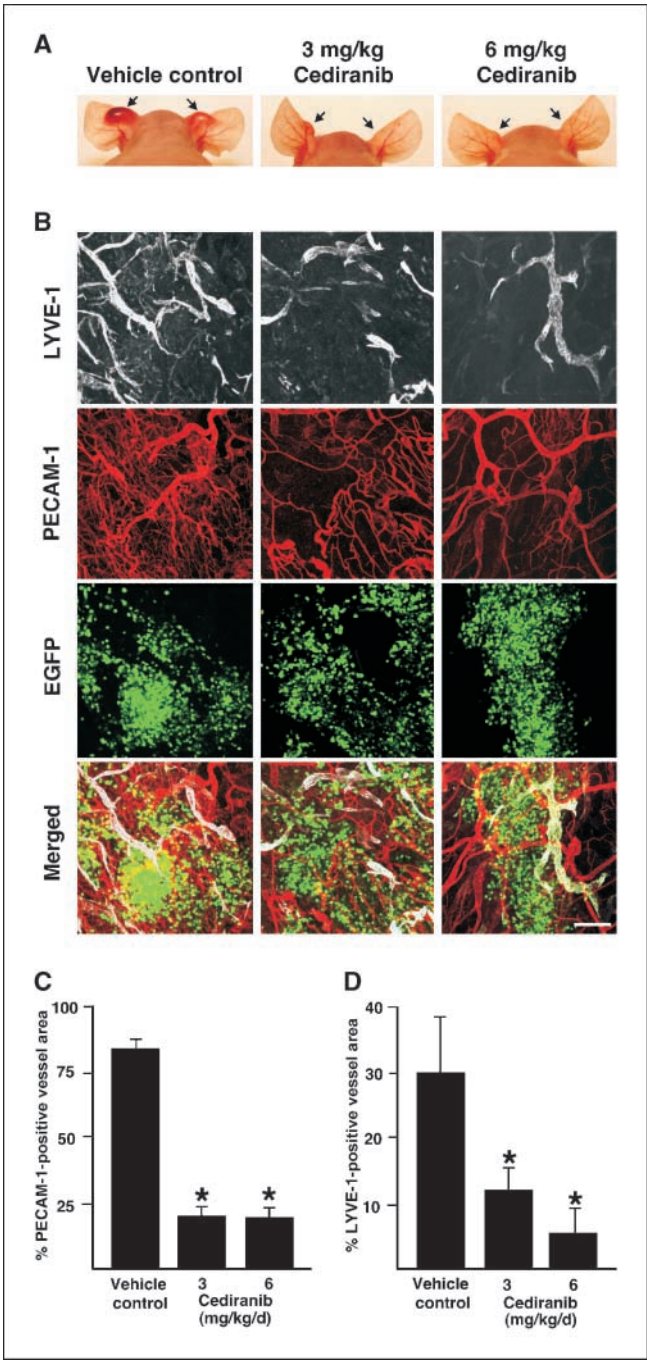

Figure 5. Cediranib inhibits tumor cell–induced angiogenesis and lymphangiogenesis. *A*, VEGF-C–expressing LNM35 tumor cells implanted into the ears of nude mice grew robustly in control vehicle-treated mice, but remained small in mice treated with cediranib (3 or 6 mg/kg/d). *Arrows,* tumors. *B,* whole mount staining for lymphatic vessels (LYVE-1, *white*) and blood vessels (PECAM-1, *red*) along with visualization of the EGFP-tagged LNM35 cells (EGFP, *green*) showed that treatment with cediranib effectively reduced tumor-associated angiogenesis and lymphangiogenesis. The white line represents 200 μm. *C,* quantification of the PECAM-1–positive vessel area. *D,* quantification of the LYVE-1–positive vessel area. *C* and *D,* asterisks indicate a statistically significant ($P \leq 0.05$) decrease in vessel density compared with the untreated controls.

VEGFR-2 have been shown to mediate specific functions (4). Therefore, one could speculate that those residues mediating migratory activity could be more sensitive to cediranib treatment. Together, however, these results do show that cediranib can comparably inhibit LEC and BEC functional activity induced by VEGFR-2–specific or VEGFR-3–specific signaling.

In vivo, VEGF-E stimulation of VEGFR-2 results in increased vascularization, which we observed as an increase in blood vessel number, as well as size. This was similar to changes in the blood vasculature induced by VEGF-A. Examination of the lymphatic vasculature, however, showed that, whereas VEGF-A clearly induced lymphatic sprouting, this was not observed with VEGF-E. Rather than an obvious induction of lymphatic sprouting, VEGF-E resulted in slightly enlarged lymphatic vessels. This effect is likely due to VEGFR-2 signaling rather than signaling through VEGFR-3, as anti-VEGFR-2, but not anti-VEGFR-3 treatment, is capable of preventing VEGF-E–induced lymphatic hyperplasia (35). Whereas the lymphatic hyperplasia induced by VEGF-E may be a secondary result of the newly formed, leaky blood vasculature, it is also possible that VEGF-E directly activates VEGFR-2 signaling on the lymphatic endothelium, as suggested by the ability of VEGF-E to induce LEC proliferation and by the ability of VEGF-A to induce lymphangiogenesis, as observed in this and other studies (48, 49). Moreover, the extent of vessel leakage induced by VEGF-E was small compared with that induced by VEGF-A. Similar to the effects of VEGF-C, VEGF-C156S was capable of inducing lymphangiogenesis *in vivo* as apparent with lymphatic vessel sprouting and the disorganized and hyperplastic appearance of the lymphatic vasculature. Whereas VEGF-C156S had no apparent effect on blood vessel growth, a slight increase in PECAM-1–positive staining was observed with the VEGF-C adenovirus. This may have been due to VEGF-C activation of VEGFR-2 on the blood endothelium and was inhibited by cediranib treatment. Furthermore, a once-daily treatment with cediranib was sufficient to block VEGF-A–induced and VEGF-E–induced neovascularization, as well as VEGF-A–induced, VEGF-C–induced, and VEGF-C156S–induced lymphangiogenesis. These results suggest that physiologic and pathologic processes dependent on VEGFR-2–driven or VEGFR-3–driven angiogenesis or lymphangiogenesis would be equally affected by cediranib treatment.

Both physiologic and pathologic angiogenesis are accompanied by an increase in vessel permeability (50). VEGF-A is a strong inducer of vascular permeability, and reports have shown that this activity can be mediated by VEGFR-1 and VEGFR-2 (51, 52). In contrast, previous studies have shown that VEGFR-3 has little to no effect on vessel permeability (33, 36). While we only observed a small increase in vessel leakage with the VEGF-E expressing adenovirus compared with the control, cediranib nevertheless reduced the effects of VEGF-E, as well as those observed with VEGF-A. Although the effect of cediranib on VEGFR-1 signaling in a cellular context has not yet been investigated, it is likely that cediranib also compromised VEGFR-1 functions mediated by VEGF-A.

Using the human lung carcinoma cell line LNM35, we observed the formation of both blood and lymphatic vessels around these VEGF-C–expressing tumor cells. Treatment with a low dose of cediranib was sufficient to compromise the growth of both tumor-associated blood and lymphatic vessels. In addition, the growth of tumors in cediranib-treated animals was impaired. As cediranib affects signaling from other kinases, in particular c-Kit, it was possible that cediranib directly affected LNM35 cell growth. In line with findings with other tumor cells (16, 17), LNM35 proliferation was only affected by cediranib at concentrations of >5 μmol/L, which is 5,000-fold greater than that needed for inhibition of ligand-induced endothelial cell proliferation. The ability of

cediranib to inhibit angiogenesis and lymphangiogenesis induced by LNM35 cells was therefore likely a result of direct effects on endothelial cells rather than indirectly through inhibition of tumor cell growth while the effects on tumor growth were more likely mediated by the ability of the inhibitor to prevent the tumors from becoming well vascularized.

Tumor progression involves a number of processes, each of which may be mediated by a number of molecules. Although therapies targeting specific ligands are advantageous for selectivity, processes, such as tumor-induced neovascularization, are complex and may require inhibition of more than one molecule. Tumor blood vessels, for example, often express both VEGFR-2 and VEGFR-3. Therefore, inhibition of either receptor tyrosine kinase alone may not be sufficient to completely inhibit tumor-induced angiogenesis. Similarly, targeting both VEGFR-2 and VEGFR-3 is likely a more potent strategy for inhibiting tumor-associated lymphatic vessel growth. Small molecular tyrosine kinase inhibitors, such as cediranib, are therefore attractive for their ability to block activity of multiple VEGFRs. Here, we have shown that cediranib inhibited VEGFR-2–induced and VEGFR-3–induced activity and functions and can block angiogenesis and lymphangiogenesis *in vivo* at similar doses. These results indicate that cediranib may prove beneficial in the clinic not only by inhibiting new tumor blood vessel growth, but may also help prevent further cancer cell dissemination by simultaneously inhibiting tumor-associated lymphangiogenesis.

Disclosure of Potential Conflicts of Interest

S.R. Wedge: AstraZeneca employee, related patents; J.M. Jürgensmeier: AstraZeneca employee. The other authors disclosed no potential conflicts of interest.

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