Assessing the Activity of Cediranib, a VEGFR-2/3 Tyrosine Kinase Inhibitor, against VEGFR-1 and Members of the Structurally Related PDGFR Family

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Abstract

Cediranib is a potent inhibitor of the VEGF receptor (VEGFR)-2 and VEGFR-3 tyrosine kinases. This study assessed the activity of cediranib against the VEGFR-1 tyrosine kinase and the platelet-derived growth factor receptor (PDGFR)-associated kinases c-Kit, PDGFR-α, and PDGFR-β. Cediranib inhibited VEGF-A-stimulated VEGFR-1 activation in AGI-G1-Flt1 cells (IC50 = 1.2 nmol/L). VEGF-A induced greatest phosphorylation of VEGFR-1 at tyrosine residues Y1048 and Y1053; this was reversed by cediranib. Potency against VEGFR-1 was comparable with that previously observed versus VEGFR-2 and VEGFR-3. Cediranib also showed significant activity against wild-type c-Kit in cellular phosphorylation assays (IC50 = 1–3 nmol/L) and in a stem cell factor–induced proliferation assay (IC50 = 13 nmol/L). Furthermore, phosphorylation of wild-type c-Kit in NCCH26 tumor xenografts was reduced markedly following oral administration of cediranib (≥1.5 mg/kg/d) to tumor-bearing nude mice. The activity of cediranib against PDGFR-β and PDGFR-α was studied in tumor cell lines, vascular smooth muscle cells (VSMC), and a fibroblast line using PDGF-AA and PDGF-BB ligands. Both receptor phosphorylation (IC50 = 12–32 nmol/L) and PDGF-BB–stimulated cellular proliferation (IC50 = 32 nmol/L in human VSMCs; 64 nmol/L in osteosarcoma cells) were inhibited. In vivo, ligand-induced PDGFR-β phosphorylation in murine lung tissue was inhibited by 55% following treatment with cediranib at 6 mg/kg but not at 3 mg/kg or less. In contrast, in C6 rat glial tumor xenografts, ligand-induced phosphorylation of both PDGFR-α and PDGFR-β was reduced by 46% to 61% with 0.75 mg/kg cediranib. Additional selectivity was shown versus Flt-3, CSF-1R, EGFR, FGFR1, and FGFR4. Collectively, these data indicate that cediranib is a potent pan-VEGFR kinase inhibitor with similar activity against c-Kit but is significantly less potent than PDGFR-α and PDGFR-β. Mol Cancer Ther; 10(5); 861–73. ©2011 AACR.

Introduction

Inhibition of VEGF-A–mediated signaling provides therapeutic benefit in oncology as monotherapy in the treatment of renal cell cancer (1, 2) and, when combined with certain cytotoxic therapies, in other disease settings (3). Approaches used to inhibit VEGF receptor (VEGFR) signaling include small molecule inhibitors that bind into the ATP-binding pocket within the kinase domain of VEGFR-2 to prevent ATP catalysis and propagation of receptor signaling. This receptor predominantly transduces the angiogenic and permeability activity of VEGF-A. Two other VEGF receptors are also described with differential binding of VEGF family ligands. Each small molecule differs in its potency against the VEGF receptors, selectivity versus other kinases, physicochemical properties, and pharmacokinetic profile (4, 5). Some VEGF tyrosine kinase inhibitors have additional activity against 1 or more members of the platelet-derived growth factor receptor (PDGFR) family of kinases (class III), which comprises PDGFR-α, PDGFR-β, c-Kit, colony-stimulating factor receptor-1, and Flt-3; these receptors have some structural homology with the VEGFR family members in that each harbors a kinase-insert sequence in its intracellular domain (6). However, VEGFR kinase...
inhibitors have also been described with activity against tyrosine kinases outside the PDGFR class and, in some cases, against serine/threonine kinases. Inhibiting multiple kinases simultaneously may provide additional therapeutic opportunities in defined disease settings but may impact adversely on tolerability, particularly if chronic administration or usage in combination with concurrent cytotoxic treatment is required.

Cediranib has been previously shown to be a potent inhibitor of VEGFR-2 and VEGFR-3 signaling in cellular assays and to inhibit the growth of both angiogenic blood vessels and lymphangiogenic vessels in vivo (7–9). In recombinant kinase assays, cediranib also inhibits VEGFR-1 kinase activity (IC_{50} = 5 nmol/L) within a similar concentration range to VEGFR-2 and VEGFR-3 (IC_{50} values of <0.1 and £3 nmol/L, respectively; ref. 7). However, a more quantitative assessment of inhibitor activity against endogenous VEGFR-1 kinase has proven technically challenging in endothelial cells because of the low intrinsic activity of this receptor. Cediranib shows selectivity against other kinases (7). Cediranib has similar potency against c-Kit when compared with VEGFR-2 in phosphorylation assays but less potency against PDGFR-α and PDGFR-β, particularly in a PDGF-AA/PDGFR-α-driven tumor cell proliferation assay. However, the activity of cediranib against a c-Kit-driven phenotypic endpoint, PDGFR-α and -β signaling in normal cell types (which may also influence therapeutic response), or a comparative inhibition of these targets in vivo was not previously examined.

This study primarily aimed to further describe the pharmacology of cediranib by (i) determining activity against VEGFR-1 in cells, (ii) using a wider complement of cell lines and assays to examine activity against c-Kit and PDGFR-α/β signaling in vitro, and (iii) to examine pharmacodynamic inhibition of c-Kit, PDGFR-α, and PDGFR-β in vivo over a dose range in which cediranib has previously shown activity in tumor models. The data confirm that cediranib is primarily a pan-VEGFR inhibitor that can inhibit wild-type c-Kit. The data also suggest that cediranib may have some partial pharmacodynamic activity against PDGFR-α and PDGFR-β receptor activation in tumors, although this inhibition may be of limited functional relevance but does inhibit FGFR1 and FGFR4.

Materials and Methods

Reagents

Cediranib [4-fluoro-2-methyl-1H-indol-5-yl]oxy]-6-methoxy-7-(3-pyrorolidin-1-ylpropoxy)quinazoline (AZD2171; ref. Fig. 1) was synthesized according to the processes described in WO 00/47212, in particular those described in example 240 of WO/47212. The free base of cediranib was used in these preclinical studies, with a molecular weight of 450.51. For all in vitro assays, cediranib was prepared initially as a 10 mmol/L stock solution in dimethyl sulfoxide (DMSO) and diluted in the relevant assay media, such that the final concentration of DMSO did not exceed 0.01%, with the exception of studies examining direct effects on tumor cells in which 1% DMSO was required to examine higher concentrations of cediranib. All in vivo studies were conducted by once-daily oral gavage. For studies in mice, cediranib was suspended in 1% (w/v) aqueous polysorbate 80 [polyoxyethylene (20) sorbitan monooleate in deionized water] and dosed at 0.1 mL/10 g of body weight.

Cell culture

NCI-H526 [a human small cell lung cancer (SCLC) line], U118MG (a human glioblastoma line), MG63 (a human osteosarcoma line), and C6 (a rat glial line) cells were purchased from the American Type Culture Collection; no further authentication was done. The M07e [a human acute myelogenous leukemia (AML) line] cells were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures); no further authentication was done. NIH 3T3 (mouse fibroblast) cells were obtained from A. Wong, Jefferson Cancer Institute; no further authentication was done. Human aortic and coronary vascular smooth muscle cells (VSMC) were purchased from PromoCell GmbH. All cell lines were routinely passaged less than 10 times with the exception of the primary vascular cells, which were passaged no more than 4 times. NCI-H526, U118MG, MG63, C6, M07e, NIH 3T3, human aortic, and coronary VSMCs were maintained in culture as per providers’ recommendation. M07e cells were maintained in culture in the presence of interleukin-3 (5 ng/mL) and granulocyte macrophage colony stimulating factor (5 ng/mL).

Inhibition of growth factor–stimulated receptor phosphorylation in vitro

The ability of cediranib to inhibit receptor phosphorylation in cells was determined using Western blotting. Cells were serum starved overnight in the presence (M07e and NCI-H526) or absence (aortic and coronary VSMC) of 0.1% bovine serum albumin or in the presence of 1% charcoal-stripped serum (MG63, U118MG, C6, and NIH 3T3 cells). Cells were then incubated with cediranib for 60 to 120 minutes and stimulated with the relevant ligand: stem cell factor (SCF; 50 ng/mL) and PDGF-AA or -BB.
PDGF-BB (50 ng/mL) for 5 to 10 minutes. SCF was obtained from R&D Systems and PDGF-AA and PDGF-BB from Sigma-Aldrich. Cell lysates of NCI-H526, M07e, and aortic and coronary VSMCs were prepared in lysis buffer 1 (50 mmol/L Tris-HCl, pH 7.6; 137 mmol/L sodium chloride; 10% glycerol; 0.1% Igepal; 0.1% SDS; 50 mmol/L sodium fluoride; 1 mmol/L sodium orthovanadate and cocktail protease inhibitor tablets (Roche Diagnostics Ltd.). Cell lysates of MG63, U118MG, C6, and NIH 3T3 cells were prepared in lysis buffer 2 (10% glycerol, 2% SDS, 50 mmol/L Tris-HCl, 200 mmol/L 2-mercaptoethanol).

The protein concentration in the lysates was determined using a bichinonic acid assay kit (Pierce) and confirmed by immunoblotting with SC316.

Sample preparation and mass spectrometry for identification of phosphotyrosine modification of VEGFR-1

Analysis of VEGFR-1–phosphorylated epitope changes was done using Cell Signaling Technology’s proprietary PhosphoScan methodology. AG1-G1-Flt-1 cells were placed in serum-free media overnight and stimulated with VEGF (50 ng/mL) or placenta growth factor (PIGF; 100 ng/mL; R&D Systems) for 5 minutes [the former also with and without cediranib (100 nmol/L; 85 minutes preincubation and 5 minutes coincubation with ligand)]. Protein extracts from AG1-G1-Flt1 cells were prepared by suspending cells in Urea Lysis Buffer (20 mmol/L HEPES, pH 8.0, 9.0 mol/L urea, 1 mmol/L β-glycerol phosphate). Lysates generated from approximately 2 × 10⁶ cells were prepared for each sample condition (control, VEGF-treated, PIGF-treated, and VEGF + cediranib-treated). The resulting protein extracts (40 mg total protein each) were then reduced with dithiothreitol (4.5 mmol/L), carboxamidomethylated using iodoacetamide (10.0 mmol/L), and subsequently digested with trypsin (1:100 weight, trypsin to total protein). Peptides were separated from nonpeptide material by solid-phase extraction with Sep-Pak C18 cartridges. Lyophilized peptides were redissolved, and phosphorylated peptides were isolated using a slurry of Magic C18 AQ reversed-phase resin. The material by solid-phase extraction was then digested with trypsin, and the resulting peptides were concentrated with PerfectPure C18 tips immediately before liquid chromatography/mass spectrometry (LC/MS) analysis. Peptides were loaded directly onto a 10 cm C18 packed with Magic C18 AQ reversed-phase resin. The column was developed with a 45-minute linear gradient of acetonitrile in 0.125% formic acid delivered at 400 μL/min. Tandem mass spectra were collected with a linear trap quadrupole (LTQ)-orbitrap hybrid mass spectrometer, using a top-ten method, a dynamic
exclusion repeat count of 1, and a repeat duration of 30 seconds. MS spectra were collected in the orbitrap component of the mass spectrometer, and MS/MS spectra were collected in the LTQ. MS/MS spectra were evaluated using TurboSequest in the Proteomics Browser package (v. 27, rev. 13). The ratios of the integrated peak height intensities for phosphopeptide quantification were obtained using the XCalibur software 2.0.7 (ThermoFinnigan). A reduction in peak intensity in VEGF-treated cells compared with control, PlGF-treated cells compared with control, or VEGF plus cediranib-treated cells compared with control was expressed as a negative fold change value. All integrated peak intensity calculations were manually reviewed to ensure proper integration of consistently shaped, coeluting peaks. Changes in phosphorylated peptide levels were measured by taking the ratio of raw intensities between control and treated cells, with the untreated sample as the reference (denominator) in each case. Raw intensity ratios were normalized using a median adjustment technique whereby the log2 ratios comprising each binary comparison (inhibitor treated versus control) was independently and globally adjusted such that the normalized median log2 ratio is zero. The normalized log2 ratios were then converted to their corresponding normalized fold changes.

**Inhibition of growth factor–mediated cellular proliferation**

NCI-H526 cells were used to determine the effect of cediranib on SCF-stimulated proliferation. Cells were seeded at a density of 1 x 10^5 per mL in 96-well microtiter plates in phenol red–free low-serum containing media (0.2% FBS) overnight. The following day cells were pretreated with cediranib (0.1–100 nmol/L) for 30 minutes before stimulation with 50 ng/mL SCF and then incubated for 72 hours at 37°C. Cell proliferation was determined using an XTT endpoint (Roche Diagnostics Ltd.). All assays were done in triplicate, and the mean ± SEM was calculated from 6 independent experiments. Human aortic VSMCs were used to determine the effect of cediranib on PDGF-AA–stimulated proliferation. Cells were seeded at 10,000 cells per well in 96-well plates in smooth muscle cell growth medium 2 (PromoCell GmbH) and incubated overnight at 37°C. The following day, the medium was replaced with Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco) containing 0.1% FBS, PDGF-BB (50 ng/mL), and cediranib. After 24-hour incubation, a bromodeoxyuridine (BrdU) reagent (Amersham) was added and cells were incubated for a further 24 hours at 37°C. Cells were fixed in formalin for 15 minutes, and proliferation was assessed by staining for BrdU by using the Cell Proliferation Fluorescence Kit (Amersham). Cells were imaged on the ArrayScan (Cellomics). All assays were done in triplicate, and the mean ± SEM was calculated from 3 independent experiments. MG63 cells were used to determine the effect of cediranib on PDGF-AA- and PDGF-BB–stimulated proliferation. Cells were seeded at 1,500 cells per well in 96-well plates in phenol red DMEM containing 1% charcoal-stripped serum for 24 hours at 37°C. The following day, the medium was replaced with DMEM (Sigma-Aldrich) containing PDGF-AA or PDGF-BB (50 ng/mL) and cediranib for a further 72 hours. Cell proliferation was determined as described earlier. All assays were done in triplicate, and the mean ± SEM was calculated from 3 independent experiments.

**Inhibition of receptor phosphorylation in vivo**

The activity of cediranib was evaluated in an NCI-H526 human SCLC tumor xenograft model. Tumors were implanted subcutaneously in the hind flank of female nude (nu/nu genotype) mice of at least 8 weeks of age. When tumors reached a volume of 0.36 ± 0.02 cm³, mice were randomized (8–10 per group) and dosed with cediranib (0.75–6 mg/kg/d) or vehicle administered once daily by oral gavage. Tumor volumes were assessed by bilateral Vernier caliper measurement at least twice weekly and calculated using the formula (length x width x height) x √ (length x width) x (π/6), where length was taken to be the longest diameter across the tumor and width the corresponding perpendicular. To remove any size dependency before statistical evaluation (the variation in mean tumor volume data increases proportionally with volume and is therefore disproportionate between groups), data were log-transformed before statistical evaluation by using a 1-tailed 2-sample t test. NCI-H526 xenograft tumors were evaluated for c-Kit receptor phosphorylation in vivo by using immunoprecipitation, following acute (2 days; 8 animals per group) or chronic (17 days; 3 animals per group) treatment with cediranib. Tumors were homogenized in lysis buffer I, and following a protein assay, 5 mg of protein from each sample was immunoprecipitated overnight at 4°C with anti-c-Kit–conjugated agarose beads. The immune complexes were washed, and proteins were eluted by boiling in SDS sample buffer. Standard SDS-PAGE methods were done to enable detection of total and pc-Kit, using antibodies as previously described. Protein phosphorylation was quantified using the ChemiGenius as described earlier.

The activity of cediranib was also evaluated in a C6 rat glial tumor xenograft model in mice. Cells were cultured in 199 media (Life Technologies) supplemented with 10% fetal calf serum and 2 mmol/L glutamine and maintained in 7.5% CO₂. For all tumor studies, C6 glial cells were resuspended in sterile PBS and inoculated subcutaneously (1 x 10⁵ in 100 μL) in the hind flank of male athymic mice. Tumor volumes were assessed as described earlier. Approximately 21 days postimplantation when the C6 glial tumor volume was between 0.5 and 0.8 cm³, mice were randomized into groups of 10 before treatment. Mice received a single bolus oral dose of either cediranib or vehicle control. Animals were sacrificed 4 hours post–dose administration. Five minutes before sacrifice, VEGF-A (20 μg per mouse; AstraZeneca) and PDGF-BB (75 μg per mouse; Biosource) were coadministered intravenously.
Cediranib is a potent inhibitor of VEGFR-1

To determine the potency of cediranib against VEGFR-1 in cells, a cell line stably transfected with full-length VEGFR-1 (AG1-AG-Flt-1) was used. Cediranib inhibited VEGF-A-driven VEGFR-1 phosphorylation with an IC\textsubscript{50} value of 1.2 nmol/L (Fig. 2A). This is comparable with the cellular potency versus VEGFR-2 (0.5 nmol/L; ref. 7) and VEGFR-3 (<1 nmol/L; ref. 8) and consistent with the primary pharmacology of the compound being that of a potent pan-inhibitor of VEGFR-1, VEGFR-2, and VEGFR-3 tyrosine kinase activity.

To identify the tyrosine phosphorylation sites on VEGFR-1 modulated by ligand-induced autophosphorylation and inhibition by cediranib, Phosphoscan was done on VEGFR-1 isolated from the AG1-G1 cells treated with VEGF-A and with VEGF-A in the presence of 100 nmol/L cediranib. Phosphorylated receptor was enriched via a total phospho-tyrosine immunoprecipitation. The residues phosphorylated on VEGFR-1 in each treated lysate were examined by specifically identifying phosphorylated peptides corresponding to VEGFR-1 (Fig. 2A and B; Supplementary Table S1). On stimulation with VEGF-A or PIGF, significant induction of phosphorylation of peptides incorporating tyrosine residues Y1053, Y1048/Y1053, and Y1048 was observed. Modest induction of phosphorylation was also detected at residues 794 and 1242, but the magnitude of change was lower (Fig. 2A). The pattern of ligand-induced phosphorylation by both VEGF-A and PIGF was similar, although the magnitude of induction was higher with VEGF-A than with PIGF. Serine-phosphorylated peptides were also detected, although the significance of these modifications is unclear (Supplementary Table S1). This shows that under these conditions, the phosphorylation status of VEGFR-1 is dynamically regulated on a restricted number of residues on engaging VEGF-A or PIGF, with Y1048 and Y1053 showing the greatest fold changes.

To determine which residues were dynamically regulated by cediranib, we compared protein extracts from cells stimulated with VEGF-A with those from cells stimulated with VEGF in the presence of 100 nmol/L cediranib (Fig. 2C). There was a marked reduction in the relative abundance of peptides corresponding to Y794, Y1053, Y1053/Y1048, and Y1048 in cediranib-treated samples, with a 37-fold reduction in the presence of the peptide corresponding to pY1053/48 in the cediranib-treated samples (Fig. 2C). The total tyrosine phosphorylation status of VEGFR-1 in the lysates used for this specific analysis was also assessed by ELISA. VEGFR-1 from each lysate was captured, and the level of tyrosine phosphorylation was detected using an antiphosphotyrosine antibody (Fig. 2D). Both VEGF-A and PIGF induced significant phosphorylation of VEGFR-1 in the lysates. Cediranib inhibited the VEGF-A-induced phosphorylation of VEGFR-1.

Cediranib inhibits c-Kit phosphorylation and SCF-induced proliferation

Cediranib inhibits c-Kit with a similar potency to that with which it inhibits the tyrosine kinase activity of VEGFRs (7). The activity of cediranib against c-Kit was tested in 2 cell lines, M07e and NCI-H526. SCF-stimulated c-Kit phosphorylation was inhibited with IC\textsubscript{50} values of 3 and 1 nmol/L, respectively (Fig. 3A and B). MAPK as a downstream signaling marker was also inhibited with an IC\textsubscript{50} value similar to that for inhibition of receptor phosphorylation (Fig. 3A and B). The relationship between inhibition of acute ligand-induced phosphorylation and SCF-stimulated c-Kit-dependent proliferation was determined using NCI-H526 cells. Cediranib inhibited SCF-stimulated proliferation of NCI-H526 cells after 72 hours with an IC\textsubscript{50} value of 0.013 nmol/L (Fig. 3C), and full inhibition being achieved at concentrations between 20 and 50 nmol/L. From these experiments, it seems that about 10-fold higher concentrations were required to inhibit functional consequences of c-Kit signaling (i.e., c-Kit–dependent cellular proliferation) than for the inhibition of receptor phosphorylation.

Mutations in c-Kit are associated with certain tumors such as gastrointestinal stromal tumors (GIST) and AML in which they drive tumor growth. The activity of cediranib against a range of common c-Kit mutations was also determined using a panel of cell lines that either expressed mutated c-Kit endogenously or were transiently transfected with mutated receptors. The c-Kit mutations assessed were V560G, V559D, W557R, D816V, T670I, D816Y, V559D, W557R, Del 557–558, V654A, T670I, D816V, D816Y, and N822K. To assess the potential of the compound to inhibit phosphorylation of these receptors, cells were incubated in the presence and absence of 20 nmol/L of cediranib. Cediranib inhibited phosphorylation of c-Kit mutants V560G, V559D, W557R, and Del 557–558, V654A, and N822K markedly, but it did not inhibit constitutive phosphorylation of c-Kit mutants T670I, D816V, and D816Y (Supplementary Table S2).

Inhibition of c-Kit phosphorylation by cediranib in vivo

Inhibition of c-Kit phosphorylation was examined in vivo in established NCI-H526 tumor xenografts, following...
chonic once-daily dosing of cediranib (17 doses of 6, 3, 1.5, or 0.75 mg/kg) to tumor-bearing mice (Fig. 3D). The dose range examined has been previously determined to result in dose-dependent inhibition of a wide range of human tumor xenograft models that do not express or have a dependency on c-Kit. C-Kit was immunoprecipitated, and the samples were analyzed for phosphorylated and total receptors. In NCI-H526 tumors, cediranib reduced phosphorylation of the receptor by greater than 80% at doses as low as 0.75 mg/kg. Although NCI-H526 tumor growth has been suggested to be dependent on c-Kit (11), despite the tumors expressing constitutively pc-Kit, no enhanced effects on growth or survival of the tumor cells were observed in these experiments (Supplementary Fig. S1) compared with other xenografts not expressing c-Kit.

Cediranib inhibits PDGFR-mediated autophosphorylation and PDGF-driven proliferation at higher concentrations

In recombinant kinase assays, cediranib has been previously shown to exhibit lower potency, 10- and 36-fold for inhibition of PDGFR-α and PDGFR-β than for VEGFRs or 2.5- and 19-fold for c-Kit (7). The activity against PDGFR-α and PDGFR-β signaling was further explored using a range of cell types including other tumor cells, VSMCs, and fibroblasts (Fig. 4A–F). PDGF-AA and PDGF-BB ligands were used in stimulation assays, the former inducing homodimerization of PDGFR-α and the latter homodimerization of PDGFR-β and heterodimerization of PDGFR-α and PDGFR-β. U118MG human glioma cells express both human PDGFR-α and PDGFR-β. Cediranib inhibited PDGF-AA–induced phosphorylation of...
PDGFR-\(\alpha\) and PDGF-BB–induced phosphorylation of PDGFR-\(\beta\), with mean IC\(_{50}\) values of 20 and 32 nmol/L, respectively. In C6 rat glioma cells, a similar IC\(_{50}\) value of 24 nmol/L was observed versus PDGF-AA stimulation of PDGFR-\(\alpha\) (Fig. 4C). In NIH 3T3 cells (mouse fibroblast line), cediranib was slightly more potent, inhibiting PDGF-BB–mediated phosphorylation of PDGFR-\(\beta\) with an IC\(_{50}\) value of 12 nmol/L (Fig. 4D). Comparable activity was found in smooth muscle cell types. In cultured human coronary VSMCs, the primary PDGFR is PDGFR-\(\alpha\) (data not shown). Cediranib inhibited PDGF-AA–stimulated receptor phosphorylation with an IC\(_{50}\) value of 15 nmol/L (Fig. 4E). In contrast, in human aortic VSMCs, the primary PDGFR is PDGFR-\(\beta\) (data not shown). In these cells, cediranib inhibits PDGF-BB–induced phosphorylation of PDGFR-\(\beta\) with an IC\(_{50}\) value of 23 nmol/L (Fig. 4F).

To determine how effectively cediranib inhibits the functional consequences of PDGFR activation, its potency was assessed in both PDGF-AA- and PDGF-BB–driven proliferation assays. In human aortic VSMCs, cediranib inhibited PDGF-BB–stimulated proliferation after 48 hours with an IC\(_{50}\) value of 36 nmol/L (Fig. 4G), similar to the potency versus PDGFR-\(\beta\) phosphorylation in the same cells. In MG63 cells, cediranib inhibited PDGF-BB–stimulated proliferation with an IC\(_{50}\) value of 63.5 nmol/L (Fig. 4H), similar to the previously reported IC\(_{50}\) value of 40 nmol/L versus PDGF-AA-induced proliferation in the same cell line (7).

Cediranib gives differential inhibition of PDGFR signaling in C6 tumors and murine lung tissue in ligand-induced acute pharmacodynamic assays

We have previously shown time- and dose-dependent inhibition of VEGFR-2 in murine lung tissue by using a
ligand-induced pharmacodynamic assay (12). This approach was taken because the interanimal variability in pVEGFR-2 levels was high, making accurate assessment of inhibitor dose responses extremely difficult. The addition of exogenous ligand to stimulate receptor phosphorylation overcame this issue. Here we used a similar approach to assess the inhibition of PDGFR activation relative to VEGFR-2 to gain greater insight into the effects on...
of cediranib on these receptors in vivo. The relative potency of cediranib versus VEGFR-2 and PDGFR-β was compared directly in vivo in the same animal. To normalize levels of pVEGFR-2 and stimulate PDGFR-α and PDGFR-β phosphorylation, animals were injected with both VEGF-A and PDGFB-B immediately before sacrifice. Lungs from animals bearing C6 tumors receiving cediranib 6, 3, 1.5, or 0.75 mg/kg for 4 hours were sacrificed. Lungs from animals bearing C6 tumors received with both VEGF-A and PDGFB-B immediately before sacrifice. Consistent with previous data (12), VEGFR-2 phosphorylation in lung was significantly reduced over a range of doses from 6 mg/kg down to 0.75 mg/kg (Fig. 5A). In contrast, phosphorylation of PDGFR-β was only significantly inhibited in lung at 6 mg/kg, with no significant inhibition achieved at lower doses (Fig. 5B).

To further explore the relative activity against activity PDGFR-α and PDGFR-β expressed in tumor cells, we examined C6 tumor xenografts. C6 cells express both PDGFR-α and PDGFR-β. Although PDGFR-α is constitutively phosphorylated in C6 cells in vivo, the injection of PDGFB-B results in the additional phosphorylation of PDGFR-β, enabling inhibition of both receptors to be studied in the same tumor. In separate studies (acute and chronic), we have assessed the activity of cediranib against PDGFR-α in C6 tumor xenografts (at 6 hours) and established that cediranib gives up to 60% inhibition of PDGFR-α phosphorylation. Again because of variability in phosphorylation between tumors, these data were variable (data not shown). Adopting an acute ligand stimulation approach allowed us to further examine inhibition of both PDGFRs in tumor and lung within the individual animals. In contrast to the modulation of receptor phosphorylation in lung tissue, phosphorylation of both PDGFR-α (Fig. 5C) and PDGFR-β (Fig. 5D) in C6 tumors, in the same animals, was partially inhibited by all doses of cediranib examined.

**Selectivity against other tyrosine kinase receptors**

Some VEGFR tyrosine kinase inhibitors also have activity against other kinases. Selectivity of cediranib against c-metFlt-3 and CSF-1R members of the PDGFR family has been shown previously (Wedge and colleagues 2005). In addition to determining the relative activity against VEGFR-1, c-Kit, and PDGFRs, we also tested its activity against wild-type Flt-3 and fibroblast growth factor receptor (FGFR) 1 and FGFR4 (Supplementary Table S3). The activity against Flt-3 was determined using OCI-AML-5 cells stimulated with Flt-3 ligand (Flt-3L). The activity against FGFR1 and FGFR4 was determined by transient overexpressing the receptor in Cos-1 cells, which resulted in constitutive phosphorylation of the receptor. Cediranib was inactive against wild-type Flt-3 (IC50 value >1 μmol/L) and had marginal activity versus FGFR-1 and -4 (IC50 values of 0.35 and 2.17 μmol/L, respectively). These data indicate that cediranib is significantly less active against these receptors than against the VEGF receptors, or c-Kit.

**Discussion**

We have previously shown that cediranib is a potent inhibitor of VEGFR-2 and VEGFR-3 and that it reduces growth of a wide range of tumor models by targeting tumor vasculature (7, 8, 12). Cediranib has selectivity for VEGFR-2 against a wide range of kinases, including the PDGFR family members CSF-1R and Flt-3 in cellular phosphorylation assays (420- and >20,000-fold selectivity, respectively, vs. VEGFR-2; ref. 7). Here we explore the pharmacology of cediranib in more depth, examining its activity against VEGFR-1 in cells and the PDGFR family members c-Kit, PDGFR-α, and PDGFR-β in vitro and in vivo within a dose range of 0.75 to 6 mg/kg which has been routinely examined within preclinical tumor xenograft experiments.

Establishing the potency of small molecule inhibitors against VEGFR-1 signaling in ligand-induced endothelial cell assays has proven challenging due to the low intrinsic kinase activity associated with this receptor. Despite this low kinase activity, there is some evidence to implicate VEGFR-1 signaling in pathologic angiogenesis (13–15) as well as in the recruitment of macrophages and myeloid precursor cell recruitment to tumors (16–18), a process which has been linked with resistance to VEGF signaling inhibitors (19–21). The role of the VEGFR-1 kinase domain in the recruitment of bone marrow-derived cells into tumors has been confirmed using VEGFR-1 TK–/–transgenic mice (22). Consequently, concurrent inhibition of VEGFR-1 and -2 signaling may afford added therapeutic benefit. To examine inhibition of VEGFR-1, we used a cell line derived from a human benign angioma into which the full-length receptor was overexpressed by stable transfection. This cell line does not express VEGFR-2 and therefore enables a more accurate assessment of activity against VEGFR-1 by avoiding any confounders that could result from VEGFR heterodimerization. The inhibition of VEGF-induced VEGFR-1 phosphorylation by cediranib, as determined by Western blotting, was evident at a potency that is comparable with that determined against VEGFR-2 and VEGFR-3 activation in cellular assays, thereby confirming that cediranib is a pan-VEGFR kinase inhibitor. Although it is clear that VEGFR-1 does induce specific signaling (10, 23, 24), there is limited information on the residues involved. We therefore also used an MS-based method to examine the residues activated on VEGFR-1 by VEGF or PI3K in AG1-G1-Flt-1 cells and inhibition of the VEGF-induced response by cediranib. VEGF-A and PI3K were found to induce a broadly similar pattern of change in VEGFR-1 increasing the phosphorylation of tyrosine residues Y794, Y1048, Y1053, and Y1242. The greatest fold change evident was at Y1048 and Y1053, which are within the tyrosine kinase domain of the receptor. Cediranib treatment abolished all VEGF-stimulated phosphorylation on the 4 residues.
described but had greatest effect against Y1048/Y1053, the signal from this peptide sequence being reduced by nearly 37-fold when compared with the untreated control, suggesting that phosphorylation at this site was the most labile. These data contrast with previous studies that have described a number of VEGFR-1 residues in the C-terminal tail as being modulated by VEGF-A treatment, in particular Y1213 (25–27) but also Y1327 and Y1333 (27).
and Y1309 in response to PlGF stimulation (24). Although peptides indicating phosphorylation at Y1213 were detected in our study, these were not modulated by ligand activation. These differences may be attributable to the cell line examined or technical approach used. We were unable to develop a pharmacodynamic assay to measure inhibition of VEGFR-1 activity in vivo, due to both the low level of receptor phosphorylation and the inability to identify selective phosphorylation-specific antibodies to VEGFR-1. However, given the similar potency of cediranib against each VEGF receptor in cells, it would be reasonable to assume that it has the ability to inhibit VEGFR-1–driven signaling responses in vivo.

In addition to having activity against the VEGF receptors, cediranib also inhibits the kinase activity of c-Kit. Inhibition of wild-type c-Kit signaling in M07e and NCI-H526 cells prevented downstream MAPK phosphorylation. Cediranib inhibited the SCF-induced proliferation of NCI-H526 cells and reduced an associated increase in AKT phosphorylation (data not shown). However, a decrease in potency (of ~10-fold) was observed for inhibition of SCF-induced proliferation, suggesting that up to 90% of SCF signaling through c-Kit and MAPK needs to be suppressed to deliver a cytostatic effect in NCI-H526 cells. In vivo, inhibition (~85%) of the constitutive phosphorylation of c-Kit in established NCI-H526 xenograft tumors was observed after 17 days of chronically dosing cediranib at 0.75 to 6 mg/kg. This suggests that cediranib may elicit a pharmacodynamic affect great enough to influence the phenotypic consequences of c-Kit signaling in vivo, although an enhanced antitumor effect was not observed in xenografts derived from this particular tumor line.

Mutation or aberrant activation of c-Kit and its ligand SCF is associated with the progression of numerous solid and hematologic malignancies, including GIST (28), SCLC (29), and AML (30). Approximately 95% of GIST cases are positive for c-Kit, with 60% to 70% positive for the c-Kit exon 11 mutations (V560G, V564A, V599D, W557R, and Del 557–558) against which the c-Kit/PDGFR/Abl kinase inhibitor imatinib shows activity (31). More recently, secondary mutations of c-Kit have been identified that confer acquired resistance to imatinib (32, 33). Cediranib was found to inhibit phosphorylation of all of the imatinib-sensitive c-Kit mutant forms found in GIST, as well as inhibited 2 of the secondary point mutations which confer acquired resistance to imatinib (V654A and N822K; refs. 34–37). However, cediranib was not active against the T670I gatekeeper mutation in c-Kit (38) or the D816V/D816Y c-Kit mutations (35–37).

Previous data generated in cellular phosphorylation assays showed that cediranib was 10- to 16-fold less active against PDGFR-α and PDGFR-β than against VEGFR, and this margin increased to 100-fold when a comparative assessment of ligand-induced proliferation was done. The in vitro data generated with cediranib in this study against receptor phosphorylation in multiple cell types is consistent with our previous data, with IC_{50} values in the range of 15 to 32 nmol/L being slightly higher than existing data in MG63 cells (cediranib IC_{50} values of 5 and 8 nmol/L against PDGFR-α and PDGFR-β, respectively). A previously observed drop-off in potency of approximately 7-fold between phosphorylation and proliferation end points for PDGF-AA/PDGFR-α signaling in MG63 cells was also apparent in the present studies when these cells were stimulated with PDGF-BB. The activity of cediranib against PDGF-BB–induced PDGFR-β phosphorylation and cellular proliferation was more comparable in the primary VSMCs, suggesting that PDGFR-mediating signaling responses may be cell-type dependent. In vivo, cediranib inhibited PDGFR-β signaling in C6 rat tumor xenografts across the dose range examined, in contrast to an effect being evident only at a dose of 6 mg/kg in normal lung tissue. This apparent discrepancy is unlikely due to species-specificity differences, given the high degree of receptor homology between mouse and rat. Although a distribution effect cannot be ruled out, the tissue concentrations of cediranib in C6 tumors did not exceed those in normal lung tissue (data not shown), nor is the inhibition of PDGFR phosphorylation in C6 tumors due to a bystander effect that is secondary to the antivasculature effects of cediranib, because compound treatment does not affect the phosphorylation of other receptor tyrosine kinases, such as EGFR in Lovo human colorectal tumor xenografts, at doses that significantly inhibit tumor growth (data not shown). An alternative explanation for the divergent effect observed in lung and C6 tumors could relate to the differential regulation or function of PDGFR-β in these tissue compartments, the receptor driving significant cellular proliferation in C6 tumors but not in normal lung tissue.

Although cediranib inhibited PDGFR-α and PDGFR-β phosphorylation in C6 tumors, this model did not seem to have increased sensitivity to the antitumor effects of the compound. A dose of 3 mg/kg cediranib, which inhibited PDGFR-α and PDGFR-β phosphorylation by 73% and 76%, respectively, 4 hours after an acute dose, inhibited tumor growth by 52% (± 4% SEM) after 10 to 14 days of continuous once-daily dosing (data not shown), an effect not dissimilar to that observed in non–PDGFR-dependent tumor models as a consequence of inhibiting VEGF signaling (5). This finding reinforces the fact that very significant inhibition of PDGFR signaling may be required to prevent phenotypic signaling responses. The activity of cediranib against PDGFR-α and PDGFR-β would therefore not be expected to contribute significantly to an effect on tumor growth or survival, unless a tumor has a particularly high dependency on signaling from these receptors.

This work highlights the significant challenge to accurately describe the relative activity of an ATP-competitive inhibitor potant against more than 1 kinase. This requires consideration of activity at the recombinant kinase level, within multiple cellular phosphorylation and proliferation assays, and then in vivo potency against the pertinent...
kinase in target tissues. These data are all required to fully interpret observations made in preclinical models. The in vivo pharmacodynamic data that show that across the dose range cediranib is primarily a VEGF signaling inhibitor with activity against c-Kit. That a significant drop-in potency is observed between ligand-induced receptor phosphorylation and cellular proliferation for c-Kit, PDGFR-α, and PDGFR-β, but not for VEGFR-2, in endothelial cell assays (7), combined with the relative order of potency against these targets within a number of in vitro assays, suggests that cediranib is primarily a VEGFR inhibitor.

Disclosure of Potential Conflict of Interest


Acknowledgments

The authors thank the staff of CDMG, Inc., for xenograft studies, Susan Lovick for statistical analysis, Jonathan Owens for FGFR-1 and FGFR-4 assays and analyses, Jonathan Dry for mutation analysis, and James Sherwood and John Smith for c-kit sequencing and mutation analyses.

Grant Support

The Phosphoscan analysis of VEGFR-1 was done by Cell Signaling Technology and funded by AstraZeneca.

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Received October 27, 2010; revised February 15, 2011; accepted February 23, 2011; published OnlineFirst March 25, 2011.

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Molecular Cancer Therapeutics

Assessing the Activity of Cediranib, a VEGFR-2/3 Tyrosine Kinase Inhibitor, against VEGFR-1 and Members of the Structurally Related PDGFR Family

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Mol Cancer Ther 2011;10:861-873. Published OnlineFirst March 25, 2011.

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