Antitumor and Antiangiogenic Activity of Cediranib in a Preclinical Model of Renal Cell Carcinoma

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Abstract. *Cediranib is a highly potent and selective vascular endothelial growth factor (VEGF) signaling inhibitor with activity against all three VEGF receptors (VEGFRs) that inhibits angiogenesis and growth of human tumor xenografts in vivo. The present study evaluated the antitumor and antiangiogenic activity of cediranib in the clinically relevant, murine renal cell carcinoma (RENCA) model and its biological response using VEGF and sVEGFR-2 as biomarkers. Mice were treated with cediranib (5 mg/kg/d p.o.) or vehicle for 2, 8 or 12 days and tumor volumes, microvessel density (MVD) and VEGF and sVEGFR-2 plasma concentrations were determined. Cediranib treatment (8 and 12 days) led to a significant reduction in tumor size (42-50%) and a highly significant reduction in MVD (30-55%) versus controls. After 12 days' treatment, VEGF plasma concentration increased significantly in both cediranib-treated and control animals and this increase correlated with tumor size; the cediranib group showed a more pronounced increase in VEGF but a reduced tumor volume compared with control animals. Plasma concentrations of VEGF reached a plateau in the cediranib group after 17-21 days' treatment. sVEGFR-2 concentrations significantly decreased over 12 days in controls, whereas they remained stable in cediranib-treated mice. sVEGFR-2 did not correlate with tumor volume in controls; mice treated with cediranib had lower relative VEGFR-2 plasma concentrations and tumor burdens. In conclusion, cediranib showed potent antitumor and antiangiogenic efficacy in the RENCA model. sVEGFR-2 plasma concentrations can act as a surrogate marker for antitumor activity of VEGFR signaling inhibitors.*

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New blood vessel formation (angiogenesis) is fundamental to tumor growth and spread (1). The importance of the angiogenic process in tumor growth and metastasis is now widely recognized and intensive research in recent years has resulted in a number of novel antiangiogenic agents (2). In adults, physiological angiogenesis is limited to a small number of specific processes, such as wound healing and renewal of the uterine lining (3). Tight control of this process is maintained by a balance of endogenous antiangiogenic and proangiogenic factors. Vascular endothelial growth factor (VEGF) has a key rate-limiting role in promoting tumor angiogenesis and exerts its effects by binding to one of three tyrosine kinase receptors: VEGF receptor-1 (VEGFR-1), -2 and -3. VEGFR-1 and VEGFR-2 are predominantly expressed on vascular endothelial cells, and activation of VEGFR-2 appears to be both necessary and sufficient to mediate VEGF-dependent angiogenesis (4, 5) and induction of vascular permeability (6). VEGFR-3 is predominantly expressed on lymphatic endothelial cells, but has been shown recently to be highly expressed on endothelial sprouts in tumors, supporting the idea that VEGFR-3 may also play a role in angiogenesis (7).

Targeting VEGF signaling has been shown to result in significant tumor growth delay in a wide range of animal models (8-13) and the clinical benefit of this approach has been confirmed in a number of studies (14, 15). Bevacizumab, a monoclonal VEGF-A neutralizing antibody, prolonged survival in patients with colorectal and non-small cell lung cancer in combination with cytotoxic chemotherapy (16). Antitumor activity has also been observed in renal cell carcinoma using the multitargeted kinase inhibitors, sorafenib and sunitinib, that inhibit VEGFRs and other protein kinases (14, 15).

Cediranib (Recentin™) is an oral, highly potent inhibitor of VEGF signaling that selectively inhibits VEGFR tyrosine

kinase activity associated with VEGFR-1, -2 and -3 (12). The effects of cediranib on receptor kinase activity have been assessed in a recombinant enzyme assay *versus* isolated kinases (12). Cediranib was shown to be a highly potent inhibitor of VEGFR-2 tyrosine kinase activity (IC_{50}<1nM) and demonstrated potent activity against VEGFR-1 and -3 (IC_{50}= 5 nM and ≤3 nM, respectively), with additional activity against c-Kit (IC_{50}=2 nM). In cell lines, cediranib treatment resulted in a dose-dependent inhibition of VEGF-induced VEGFR-2 phosphorylation in human umbilical vein endothelial cells (HUVECs), with an IC_{50} of 0.5 nM. Consistent with inhibition of VEGFR-2 tyrosine kinase activity, cediranib was also shown to be a potent and selective inhibitor of VEGF-stimulated HUVEC proliferation (IC_{50}=0.4 nM). Cediranib also inhibited VEGFR-3 at concentrations <1 nM and blocked proliferation of lymphatic vascular endothelial cells as well as lymphangiogenesis (17). In Calu-6 human lung tumor xenografts, cediranib led to a significant reduction in tumor vessel staining of phosphorylated VEGFR-2 and tumor microvessel density (MVD), which was detectable following 52 h of cediranib treatment (18). Cediranib has also demonstrated broad antitumor activity in a range of preclinical tumor models, *e.g.* colon, lung, prostate, glioblastoma and osteosarcoma (11, 12).

A series of phase I studies have been conducted to investigate cediranib in patients with cancer, both as monotherapy and in combination with certain other anticancer strategies (19-23). These investigations have shown cediranib to be generally well tolerated, with a side-effect profile that is tolerable and manageable. Currently available pharmacokinetic data are supportive of a once-daily oral dosing schedule for cediranib (19, 22). Furthermore, preliminary efficacy data have demonstrated that cediranib has potential antitumor activity in multiple tumor types including lung, breast, colorectal, renal, prostate and glioblastoma (19, 22-24). Furthermore, cediranib treatment resulted in a dose- and time-dependent reduction in soluble (s)VEGFR-2 plasma concentrations and acute increases in VEGF at all doses (19). Cediranib is currently in phase III development in first-line colorectal cancer and recurrent glioblastoma.

For conventional cytotoxic anticancer agents, the optimal dose has usually been defined as the maximum tolerated dose (MTD). In contrast, antiangiogenic agents may achieve maximum therapeutic effect at doses well below the MTD. Therefore, it is important to assess quantifiable effects on the molecular target or biological parameters downstream of the molecular target, as well as safety end points to establish the dose–effect relationship. Biomarkers are important to establish biological activity and determine the optimal biological dose (25). Various angiogenic factors produced by solid tumors have been identified and their roles in the formation of new blood vessels have been elucidated (26). Soluble markers of tumor angiogenesis such as VEGF, basic fibroblast growth factor (bFGF), soluble fms-like tyrosine kinase-1 (sFLT-1), soluble angiopoietin receptor sTIE-2 and sE-selectin have been investigated as potential surrogate markers of biological activity in several studies of angiogenesis inhibitors (27-29).

The orthotopic and syngeneic RENCA model is well characterized with respect to primary tumor formation in the kidney and the subsequent metastases formed in the lung (8). Antitumor efficacy has also been reported in this model (30) using vandetanib (Zactima™), an inhibitor of VEGFR-2 tyrosine kinase activity that also has additional activity against epidermal growth factor receptor tyrosine kinases (31). Further studies will be required to confirm these results in the clinical setting.

The aim of this study was to determine the antitumor and antiangiogenic activity of cediranib in the RENCA model and to evaluate its biological response using VEGF and sVEGFR-2 as biomarkers.

Materials and Methods

Compounds. Cediranib was provided by AstraZeneca (Alderley Park, Macclesfield, UK). The free base of cediranib (4-[(4-fluoro-2-methyl-1H-indol-5-yl)oxy]-6-methoxy-7-(3-(pyrrolidin-1-yl)propoxy)quinazoline; molecular weight=450.51) was used for all preclinical studies as described by Wedge *et al*. (12). Cediranib was suspended in 1% (w/v) aqueous polysorbate 80.

Cell culture. Murine RENCA cells were originally obtained from a tumor that arose spontaneously in the kidney of BALB/c mice (32). Histologically, RENCA consists of granular cell-type adenocarcinoma, which is pleomorphic with large nuclei. Monolayers of murine RENCA cells were grown in RPMI-1640 with phenol red, supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 units penicillin/ml, and 100 μg of streptomycin/ml. RENCA cells were cultured in a humidified atmosphere of 95% air and 5% carbon dioxide at 37˚C. Media were routinely changed every 3 days.

Mice. All experimental protocols were approved by the Ethics Committee for Animal Experimentation according to the UK Coordinating Committee on Cancer Research Guidelines. The experimental protocol was registered with the Regierungspräsidium Freiburg, Germany (G-04/50). All experiments were performed on 6 to 8-week-old female BALB/c mice (approximate weight, 20 g; Charles River, Sulzfeld, Germany). Mice were maintained in separated conventional housing (three mice/cage) at constant temperature and humidity. Mice were anesthetized (1.5-2.5% isoflurane with an oxygen flow of 2 l/min) and injected with 40 μl of RENCA cells (4×10^6 in phosphate-buffered saline [PBS]) into the subcapsular space of the left kidney through a flank incision. This model was first described by Murphy and colleagues (32) who showed that a subcapsular renal injection of RENCA cells in a syngeneic BALB/c mouse resulted in the progressive development of a primary tumor in the left kidney and spontaneous metastases to the regional lymph nodes, lung, peritoneum and liver (8). Animal weights were recorded three times a week and animal behavior and welfare were monitored daily.

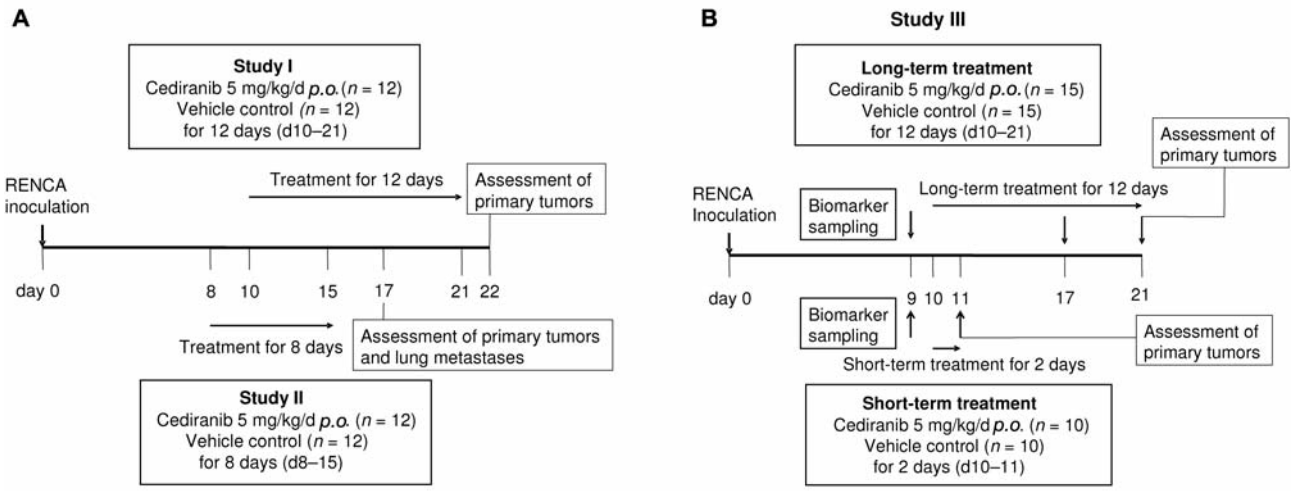

Figure 1. *A. Treatment schedule and time points for primary tumor assessment and sampling for soluble markers. Treatment of primary tumors (studies I and II); B, efficacy of short- and long-term cediranib treatment and plasma biomarker analysis (study III).*

Study design and treatments. In all studies, RENCA cells were injected into the subcapsular space of the left kidney on day 0. Mice were treated once daily by *p.o*. gavage with cediranib (5 mg/kg) or vehicle (1% [w/v] aqueous polysorbate 80). Three studies were conducted. In studies I and II, the efficacy of cediranib treatment (T) on primary tumors was assessed compared with vehicle (C) (expressed as % T/C). In study I, 24 mice were divided into two groups (n=12/group) and treated with cediranib or vehicle for 12 days (d10-21), and primary tumor assessment was performed on day 22 (Figure 1A). In a separate group of mice (study II), 24 mice were treated for 8 days (d8-15) with cediranib or vehicle (n=12/group), and assessment of primary tumors and lung metastases was performed on day 17. The length of time tumors were grown before the start of treatment differed between the two studies (12 days in study I and 8 days in study II) because each study used different RENCA sublines with different behavior. The tumor assessments were performed earlier in study II (day 17) than study 1 (day 22) because of ethical reasons (the control group became clinically symptomatic).

In study III, the effect of long-term (12 d) and short-term (2 d) cediranib treatment on the plasma biomarkers VEGF and sVEGFR-2 was assessed *versus* controls (Figure 1B). To assess the plasma concentration of these biomarkers in tumor-bearing animals, a group of nontumor-bearing untreated animals (baseline controls) were compared with tumor-bearing cediranib- and vehicle-treated mice. In study III, 65 mice were divided into five treatment groups: group 1 (baseline control; n=15) mice were sacrificed on day 12; groups 2 and 3 were treated with vehicle or cediranib, respectively on days 10-11 and were sacrificed on day 11 (n=10/group); groups 4 and 5 were treated with vehicle or cediranib, respectively on days 10-21 and were sacrificed on day 21 (n=15/group).

Tumor and lung metastases assessments. At the end of each study, mice were sacrificed and primary tumors or lungs were dissected and weighed. Tissues (primary tumor or lung) were frozen in liquid nitrogen and stored at –80˚C until further analysis. Primary tumor volume was assessed using calipers (length × width × diameter). Numbers of metastases in the lung were counted using a dissection microscope (Leica M-690, Germany; magnification ×14).

Blood sample collection for biomarker analysis. For the analysis of biomarkers (VEGF and sVEGFR-2) blood samples (50-200 µl blood) were taken from mice *via* the retroorbital vein plexus at the timepoints indicated in Figure 1B. During the blood collection, mice were anesthetized (1.5-2.5% isoflurane with an oxygen flow of 2 l/min). In the long-term treatment groups, blood sampling was performed on day 9 (1 d before start of treatment), day 17 (2 h after treatment) and at necropsy on day 21 (2 h after treatment). In the short-term treatment groups, blood sampling was performed on day 9 (1 d before treatment) and on day 11 (2 h after the second treatment; necropsy). For the baseline group (nontumor-bearing untreated animals), blood samples were taken on days 0, 8 and 12.

Determination of soluble biomarker plasma concentrations (VEGF and sVEGFR-2). Blood samples were directly collected into EDTA-coated tubes and centrifuged for 10 min at 3,500 rpm at 4˚C. The supernatant was transferred into tubes with external screw threads and stored at –20˚C until further analysis. VEGF and sVEGFR-2 concentrations were determined using the relevant quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (Quantikine Immunoassay, R&D Systems Europe, Oxford UK; mouse VEGF, #MMV00 and Mouse sVEGFR-2, #MVR200B) according to the instructions provided by the manufacturer. The VEGF ELISA recognizes both 164- and 120-amino acid isoforms of mouse VEGF. Samples were prediluted 1:3 and 1:15 for VEGF and sVEGFR-2 determination, respectively. VEGF and sVEGFR-2 plasma concentrations are displayed as absolute values (in pg/ml and ng/ml, respectively) and relative values ($\Delta VEGF=VEGF_{d21}-VEGF_{d9}$; Δ%sVEGFR-2: % change compared with initial measurement). Minimal detectable plasma concentrations were 3 pg/ml and 27 pg/ml for VEGF and VEGFR-2, respectively. The determination of VEGF and sVEGFR-2 plasma concentrations was performed in triplicate.

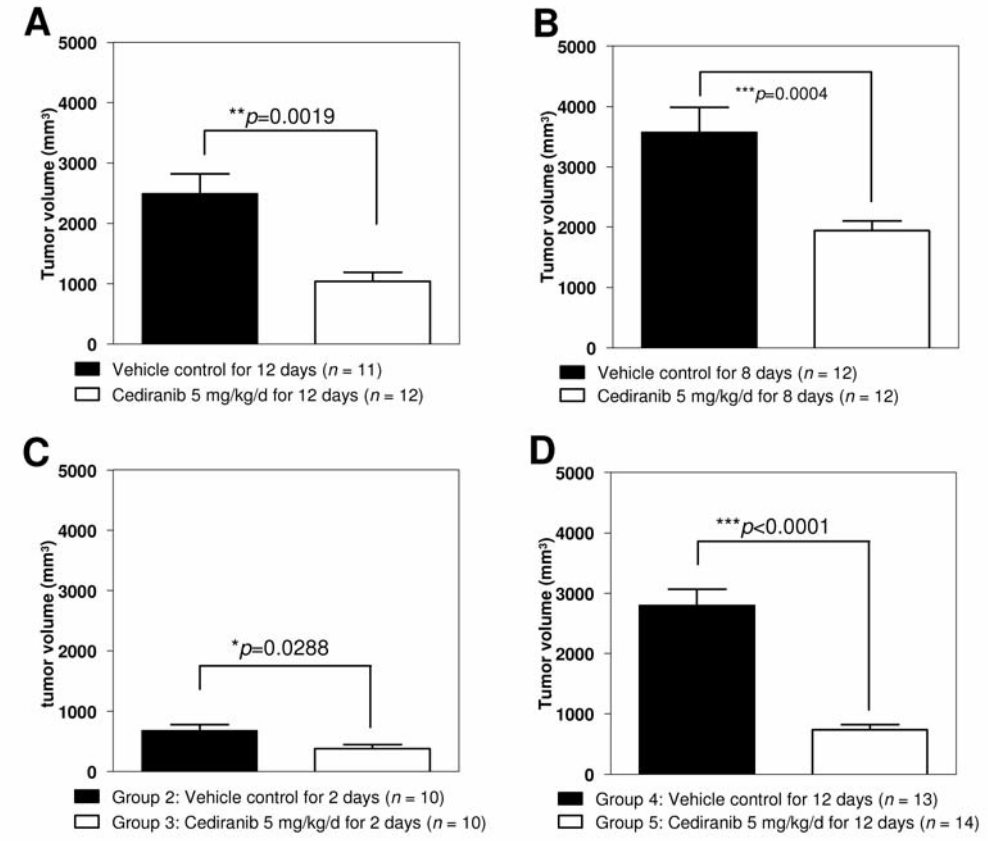

Figure 2. *Cediranib inhibits primary tumor growth in murine renal cell carcinoma. RENCA cells were inoculated into the subcapsular space of the left kidney of syngeneic BALB/c mice on day 0. Mice were treated with cediranib (5 mg/kg/d) or vehicle control for A, 12 days (d10–21); B, 8 days (d8–15); C, 2 days (d10–11); and D, 12 days (d10–21). Primary tumor volume was assessed on day 22 (A), day 17 (B), day 11 (C) and day 21 (D). Values are means; bars, SEM. P-values versus vehicle control, Mann-Whitney U test.*

Immunohistochemistry. Tumor vasculature of primary tumors and lung tissue was assessed on cryosections (5-10 μm). Blood vessels were visualized using immunohistochemical staining for CD31 (PECAM-1 and MEC 13.3; PharMingen, San Diego, CA, USA). Vessels were counted microscopically using a defined magnification (×200). Any stained endothelial cell or endothelial cell cluster, with or without a lumen that was clearly separated from adjacent microvessels was considered as a single, countable microvessel. Counting was performed on two sections in three different parts of each section, with each section being counted in duplicate. The evaluation was performed by two observers, blinded to the treatment groups. The mean vessel count (per high-power field; six areas in duplicate, n=12) was used to represent CD31 MVD.

Statistical analysis. All statistics were performed using the GraphPad Prism 5 (La Jolla, CA, USA) software package. All data are shown as means±SEM. Statistical analysis of efficacy data was carried out using the Mann-Whitney test. A p-value of <0.05 was considered statistically significant. Correlation analyses were performed using the linear regression algorithm. *P*-values were generated using the F-test. Low p-values represent a high probability that the data points represent a true change (slope≠0).

Results

Cediranib inhibits tumor growth of orthotopic murine renal cell carcinoma. Previous studies have shown that the subcapsular renal injection of RENCA cells in a BALB/c mouse is followed by the progressive development of a primary tumor mass in the left kidney. One week later, the primary tumor is usually macroscopically visible; after 10 days, spontaneous metastases develop in the regional lymph nodes, lungs, peritoneum and liver (33, 34).

To determine whether cediranib is effective in inhibiting tumor growth in the RENCA model, mice were treated once daily with 5 mg/kg cediranib *p.o.* gavage following inoculation of RENCA tumor cells into the lower pole of the left kidney. In study I, daily treatment with cediranib or vehicle control was performed for 12 days (d10-21) and primary tumor assessment was performed after 12 days of treatment (d22). The primary tumor volume in the left kidney in the cediranib-treated group was compared with the vehicle control group. Primary tumor volume in mice treated with

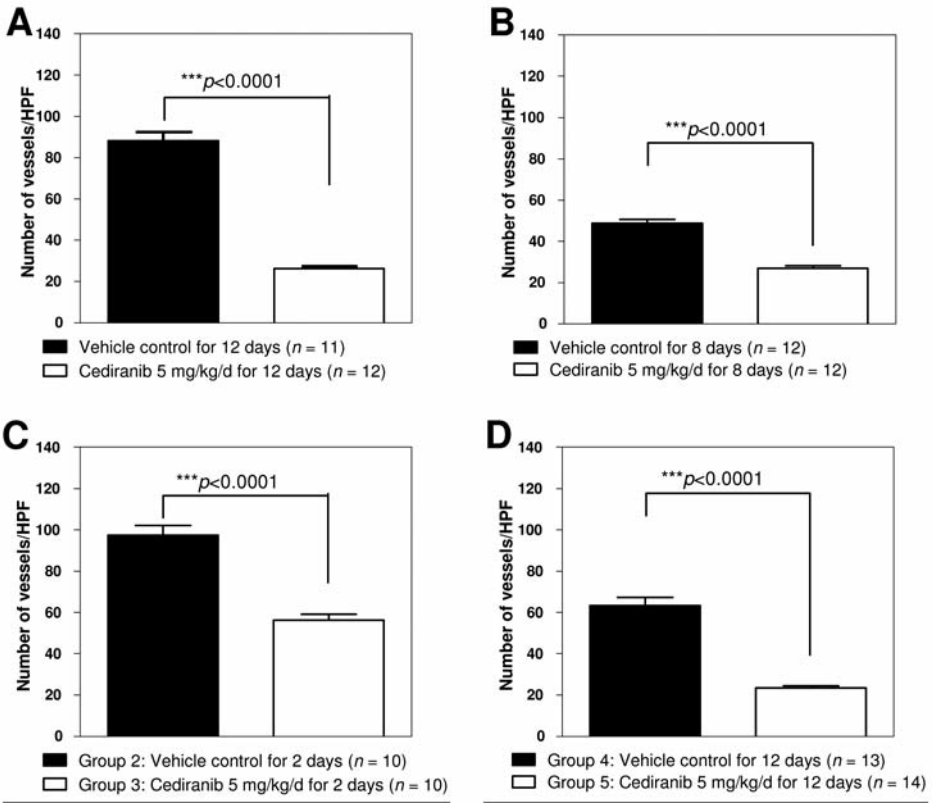

Figure 3. *Treatment with cediranib reduces vessel density in primary tumors of murine renal cell carcinoma. RENCA cells were inoculated into the subcapsular space of the left kidney of syngeneic BALB/c mice on day 0. Mice were treated with cediranib (5 mg/kg/d) or vehicle control for A, 12 days (d10–21); B, 8 days (d8–15); C, 2 days (d10–11); and D, 12 days (d10–21). Microvessel density was determined on day 22 (A), day 17 (B), day 11 (C) and day 21 (D). For visualization of the blood vessels, immunohistochemical staining for CD31 was performed. Microscopic vessel counts were performed using a ×200 magnification. Values are means of 12 single values (two slides, three sections, each assessed in duplicate); bars, SEM. P-values versus vehicle control, Mann-Whitney U test.*

cediranib for 12 days (study I) was significantly reduced by 58% (T/C 42%; p=0.0019; Figure 2A). In study II, daily treatment was performed for 8 days and cediranib significantly reduced the primary tumor volume (T/C 50%, p=0.0004; Figure 2B). In study III, animals were treated short term for 2 days (d10-11; groups 2 and 3) or longer for 12 days (d10-21; groups 4 and 5) with cediranib or vehicle control. After only 2 days of treatment cediranib reduced the primary tumor volume by 40% compared with the control (T/C 60%; p=0.0288; Figure 2C). Long-term treatment with cediranib for 12 days reduced the tumor volume by 73% (T/C 27%; p<0.0001; Figure 2D).

Cediranib was well tolerated in all of the studies and had no significant effects on body weight or the general well-being of the animals. Cediranib reduced the number of lung metastases after 8 and 12 days of treatment, although this reduction did not reach statistical significance (data not shown) owing to the high variability of the number of lung metastases in the control group. As previously described, due to the very low number of metastases in other organs (regional lymph nodes, spleen, liver) no notable differences were observed in the cediranib-treated mice (data not shown) (30).

Cediranib reduces vessel density in murine renal cell carcinoma. For histological examination of the tumor vasculature, primary tumor tissues of all treatment groups from all studies were stained for CD31. Vessel densities in primary tumors of vehicle controls were higher than those of cediranib-treated mice, reflecting a decrease in vessel density due to cediranib treatment. The vessel density in primary tumors of mice treated with cediranib (5 mg/kg/d) for 12 days in study I significantly decreased by 70.2% (T/C 29.8%; p<0.0001; Figure 3A) compared with vehicle controls. In study II, cediranib treatment for 8 days reduced MVD in primary tumors by 45% (T/C 55%; p<0.0001; Figure 3B). Both short- (2 d) and long-term (12 d) treatment with cediranib in study III significantly reduced MVD by 43% (T/C 57%; p<0.0001; Figure 3C) and by 63% (T/C 37%; p<0.0001; Figure 3D), respectively compared with vehicle-treated controls.

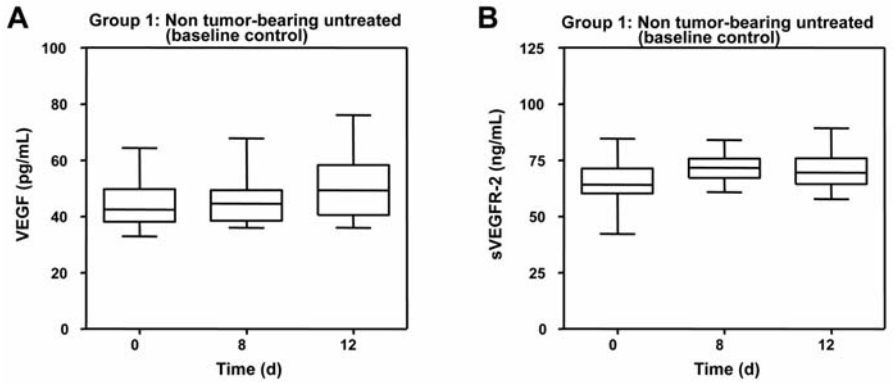

Figure 4. *Plasma VEGF and sVEGFR-2 concentrations were stable in untreated, nontumor-bearing mice. For the analysis of VEGF (A) and sVEGFR-2 (B) plasma concentrations, blood samples were taken from mice on days 0, 8 and 12. Box and whisker plots are shown, which indicate the highest and the lowest data points as well as median, 25% and 75% percentiles; n=15.*

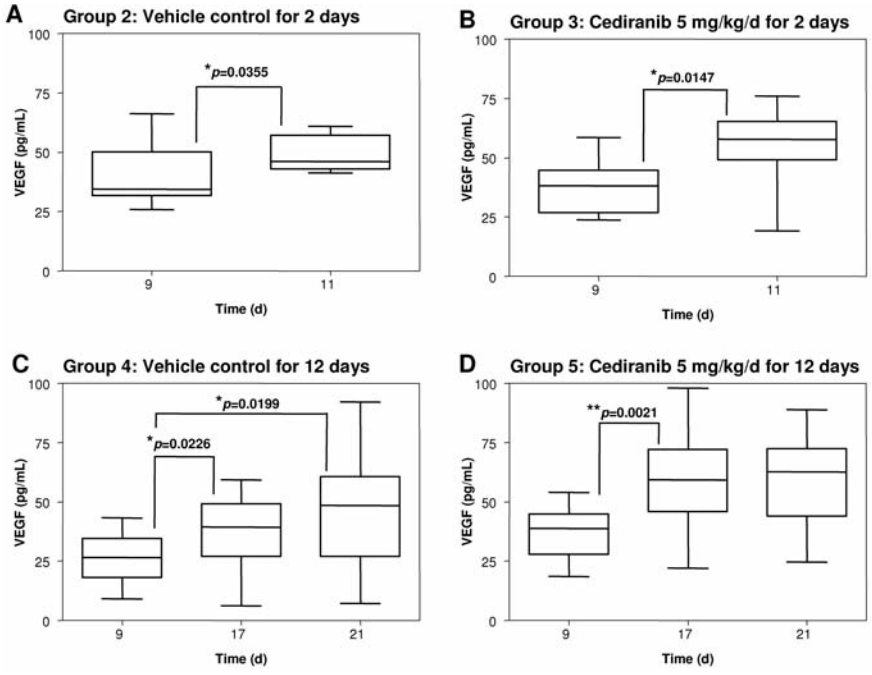

Figure 5. *VEGF plasma concentrations increase in cediranib-treated and vehicle control tumor-bearing animals. Mice were treated short-term for 2 days (d10–11) with vehicle control (A) and cediranib (5 mg/kg/d) (B); and long-term for 12 days (d10–21) with vehicle control (C) and cediranib (5 mg/kg/d) (D). Plasma VEGF concentrations were determined on days 9 and 11 (A and B) and days 9, 17 and 21 (C and D). Box and whisker plots are shown, which indicate the highest and the lowest data points as well as median, 25% and 75% percentiles. P-values versus VEGF or sVEGFR-2 plasma concentrations on day 9, Mann-Whitney U-test.*

VEGF and sVEGFR-2 plasma concentrations remain stable in nontumor-bearing untreated mice. No significant changes of VEGF and sVEGFR-2 plasma concentrations were detected over the 12-day period (d0, 8 and 12) in nontumor-bearing untreated animals (n=15; Figure 4A and B). The mean plasma concentrations of VEGF and sVEGFR-2 were 46.29 pg/ml ±9.508 (range, 32.95-76.01) and 69.66 ng/ml ±7.907 (range, 42.27-84.57), respectively over the duration of the study.

VEGF plasma concentration increased in cediranib-treated and control mice and correlated with tumor volume. Blood was taken from animals for the determination of VEGF and sVEGFR-2 plasma concentrations at different time points of

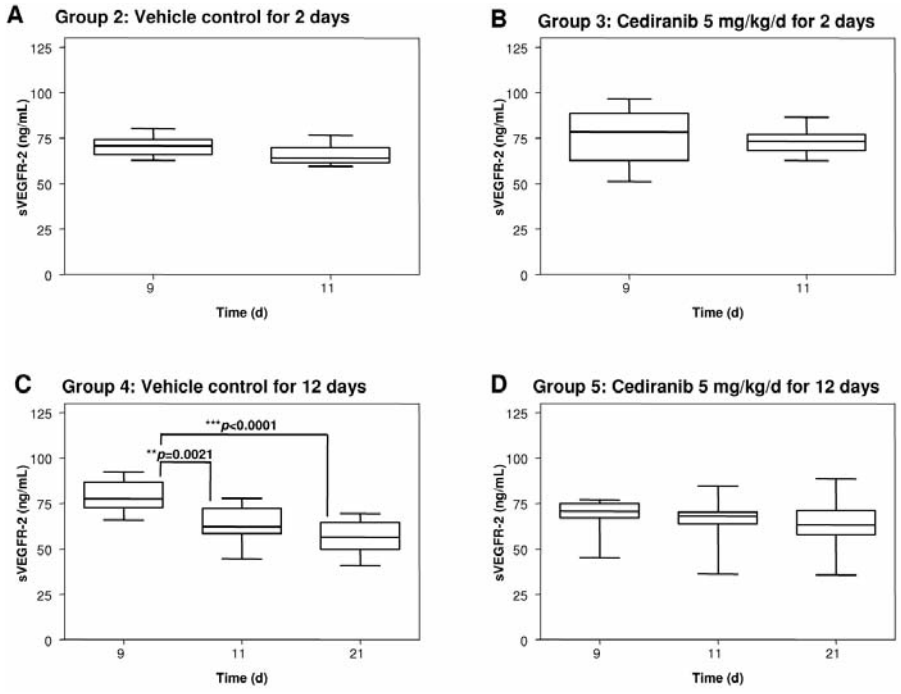

Figure 6. *Plasma concentrations of sVEGFR-2 decrease in vehicle control mice, but not in long-term cediranib-treated animals. Mice were treated short-term for 2 days (d10–11) with vehicle control (A) and cediranib (5 mg/kg/d) (B); and long-term for 12 days (d10–21) with vehicle control (C) and cediranib (5 mg/kg/d) (D). Plasma sVEGFR-2 concentrations were determined on days 9 and 11 (A and B) and days 9, 17 and 21 (C and D). Box and whisker plots are shown, which indicate the highest and the lowest data points as well as median, 25% and 75% percentiles. P-values versus VEGF or sVEGFR-2 plasma concentrations on day 9, Mann-Whitney U-test.*

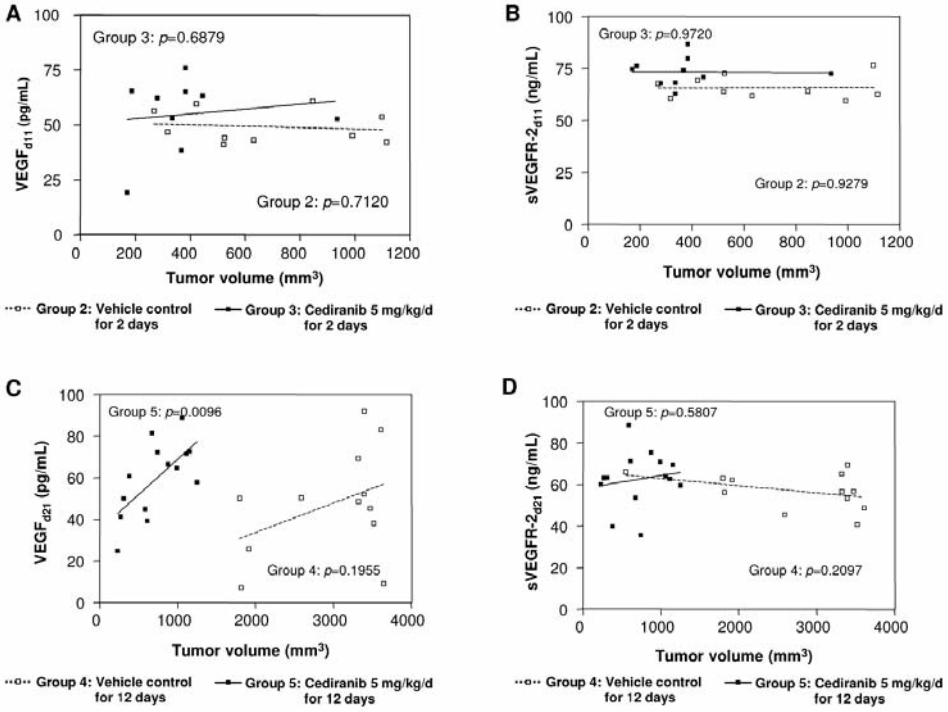

Figure 7. *Cediranib-treated animals have higher VEGF plasma concentrations but smaller tumor volumes (closed squares) than vehicle control animals (open squares) on long-term treatment. Mice were treated with cediranib (5 mg/kg/d) or with vehicle control short term for 2 days (d10–11; A and B) and long term for 12 days (d10–21; C and D). Plasma VEGF and sVEGFR-2 plasma concentrations from day 11 (A and B) and day 21 (C and D) were correlated with primary tumor volumes from day 11 (A and B) and day 21 (C and D).*

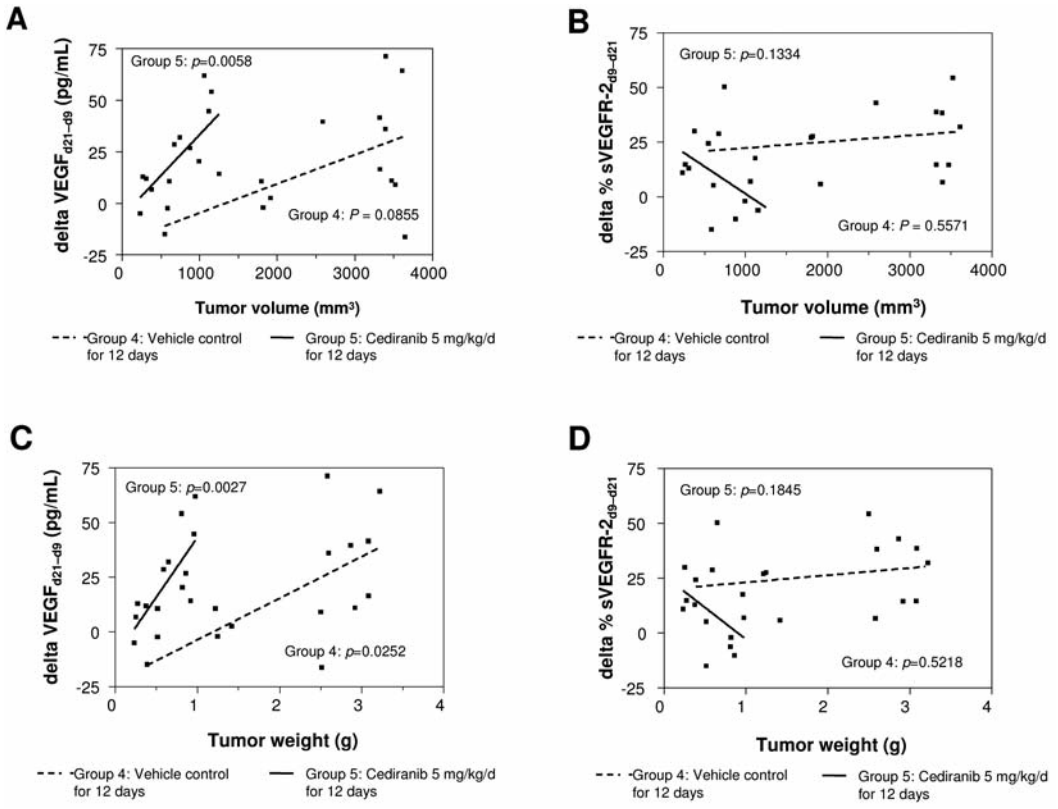

Figure 8. *Plasma concentrations of relative sVEGFR-2 (Δ% sVEGFR-2$_{d9–d21}$=% change compared to initial measurement decreased (A, B), in cediranib-treated animals, with smaller tumor volumes than vehicle control mice. Mice were treated with cediranib (5 mg/kg/d) or with vehicle control long term for 12 days (d10–21). A, VEGF and B, sVEGFR-2 plasma concentrations were correlated with primary tumor volumes from day 21. C, VEGF and D, sVEGFR-2 plasma concentrations were correlated with primary tumor weights from day 21.*

treatment with cediranib or vehicle control: short-term treatment for 2 days and long-term treatment for 12 days. VEGF and sVEGFR-2 plasma concentrations were determined 1 day before the start of treatment and 2 h after treatment on days 11 (short-term treatment), and on days 17 and 21 (long-term treatment). VEGF plasma concentrations increased significantly between days 9 and 11 in both the vehicle control group (p=0.0355; Figure 5A) and the short-term cediranib-treated group (p=0.0147; Figure 5B), with a greater increase in the cediranib-treated group compared with pre-treatment concentrations. There was no correlation between VEGF plasma concentrations and tumor volume in either group during 2 days of treatment (Figure 7A). In contrast, VEGF plasma concentrations significantly increased in vehicle-treated controls (p=0.0199; Figure 5C) and in long-term cediranib-treated mice (p=0.0021; Figure 5D) and this increase appeared to be greater in mice treated with cediranib. VEGF plasma concentrations reached a plateau following cediranib treatment between days 17 and 21 after the initial increase. Absolute and relative VEGF plasma concentrations correlated positively with tumor volume in mice treated long-term with cediranib (p=0.0096 and p=0.0058, respectively) and those treated with vehicle (p=0.1955 and p=0.0855, respectively; Figure 7C and 8A). Cediranib-treated mice had higher VEGF plasma concentrations and lower tumor volumes compared with vehicle controls (Figure 7A and 7C).

Plasma concentrations of sVEGFR-2 decrease in vehicle control mice but not in long-term cediranib-treated mice. Plasma concentrations of sVEGFR-2 were determined at the same time points as VEGF (see above and Figure 1B). sVEGFR-2 plasma concentrations were similar in control and cediranib-treated mice following short-term treatment (Figure 6A and 6B). sVEGFR-2 plasma concentrations significantly decreased in long-term control animals (p=0.0021 between d9 and 11; p<0.0001 between d9 and 21; Figure 6C), whereas cediranib-treated mice showed a slight, but not significant decrease (Figure 6D). Although there was no correlation between absolute sVEGFR-2 plasma concentrations and tumor volume following short-term and long-term treatment in both cediranib-treated and vehicle control animals (Figure

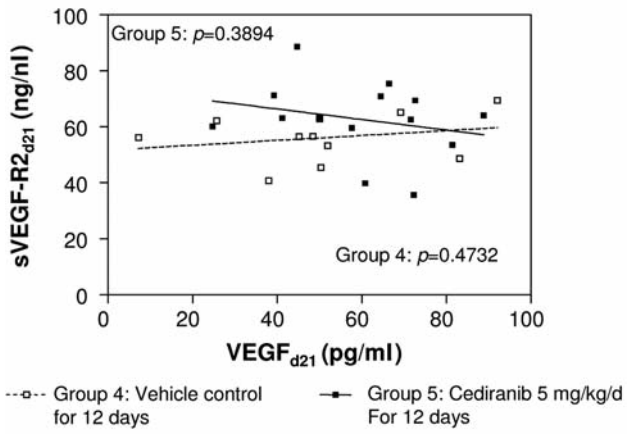

Figure 9. *No correlation of VEGF and sVEGFR-2 plasma concentrations were found in the cediranib-treated and vehicle control tumor-bearing animals. Mice were treated with cediranib (5 mg/kg/d) or with vehicle control long term for 12 days (d10–21). VEGF plasma concentrations were correlated with sVEGFR-2 plasma concentrations from day 21.*

7B and D), mice treated with cediranib showed greater reductions in relative sVEGFR-2 plasma concentrations (Δ% $sVEGFR\text{-}2_{d9–d21}$) and tumor size compared with vehicle controls (p=0.1334 *versus* p=0.5571; Figure 8B). Plasma concentrations of sVEGFR-2 showed less variability than did those of VEGF. There was no correlation between VEGF and sVEGFR-2 plasma concentrations on long-term treatment in cediranib-treated and vehicle control animals (p=0.4732 *versus* p=0.3894; Figure 9).

Discussion

Recent research in the treatment of cancer has focused on seeking novel strategies to control cell growth and metastasis. One such strategy is to target angiogenesis, and such treatment primarily exerts a cytostatic action, thereby preventing further tumor vascularization, slowing primary tumor growth and limiting the development of metastases (35). VEGF is the most important of the proangiogenic factors involved in tumor vascularization (36), and the clinical value of inhibiting VEGF signaling has now been confirmed in studies examining the anti-VEGF-A monoclonal antibody, bevacizumab (16, 37) and the small molecule tyrosine kinase inhibitors sunitinib and sorafenib.

The aim of our study was to investigate the antitumor and antiangiogenic effects of cediranib, a highly potent and selective signaling inhibitor of the VEGFR-1, -2 and -3 tyrosine kinases, in the VEGF-dependent murine renal cell carcinoma model, and to evaluate plasma concentrations of VEGF and sVEGFR-2 as potential biomarkers.

Treatment with the small-molecule tyrosine kinase inhibitor cediranib led to a significant reduction of primary tumor growth in the murine RENCA model. Cediranib treatment for 8 or 12 days reduced tumor volumes compared with tumors in vehicle-treated control mice. Moreover, this reduction in tumor size was already apparent after only 2 days of treatment with cediranib. Treatment with cediranib was well tolerated in all mice, with no drug-related weight losses or changes in behavior noted. The antitumor effects of cediranib are very likely attributable to the inhibition of blood vessel formation because a significant decrease of vessel density in primary tumors was observed. Cediranib significantly reduced vessel density after only 2 days of treatment by 43%, and thereafter by 45% (d8) and 70.2% (d12) compared with the control group, indicating that cediranib is a potent inhibitor of angiogenesis and neovascular survival; in line with data published previously in the Calu-6 tumor xenograft model (18).

These data confirm the antitumor and antiangiogenic potential of cediranib seen in other tumor models (11, 12). In a study by Wedge *et al*. (12), once-daily oral administration of cediranib (1.5-6 mg/kg/d) resulted in a dose-dependent inhibition of tumor growth in a range of histologically distinct human tumor xenografts. In each of the tumor models, treatment with the highest dose investigated (6 mg/kg/d) resulted in >90% inhibition of tumor volume compared with time-matched controls. Gomez-Rivera *et al*. (38) demonstrated that cediranib induced a significant reduction in tumor size in contrast to the control group in an orthotopic murine anaplastic thyroid cancer model. Cediranib also showed antitumor activity in pediatric tumor xenografts including rhabdoid, Wilms', Ewing's, glioblastoma, and neuroblastoma (11).

A major obstacle in the development of targeted agents, particularly those inhibiting the VEGF signaling pathway, is the lack of non-invasive biomarkers to monitor response during treatment. In the present study, VEGF and sVEGFR-2 plasma concentrations were determined at different time points using two treatment regimens: short-term for 2 days and long-term for 12 days. The nontumor-bearing untreated mice (baseline control group 1) showed no significant changes of VEGF and sVEGFR-2 plasma concentrations at three measurements over 12 days as well as low variability, indicating that the ELISA platform is a valid method for quantification of these biomarkers in BALB/c mice. VEGF plasma concentrations increased in both cediranib-treated and vehicle control animals after long-term treatment. However, in cediranib-treated mice there was a more pronounced VEGF increase compared with vehicle controls and subsequently, plasma concentrations plateaued between days 17 and 21. The correlation between VEGF plasma concentrations and tumor size in control animals suggests that the tumor cells could be a source of VEGF secretion as suggested in the literature (39). However, cediranib-treated animals exhibited higher VEGF plasma concentrations and

lower tumor volumes compared with vehicle controls. Possible explanations for the VEGF increase following cediranib treatment could be the compensation of tumor-induced hypoxia or a tumor-independent VEGF increase as a stress response to VEGF signaling, although this was not investigated in the present study.

In previous clinical studies, plasma concentrations of VEGF have been evaluated before and during treatment, and have yielded conflicting reports (26, 40-42). Interestingly, in a preclinical model, an increase in VEGF after treatment of either tumor-bearing or nontumor-bearing mice with a VEGFR-2 blocking monoclonal antibody, but not with the small-molecule VEGFR-2 inhibitors PTK787/ZK 222584 and SU5416, was reported, implying that VEGF signaling blockade at the level of the ligand *versus* the receptor can elicit different responses (43). However, in the latter study by Bocci *et al*., the VEGFR-2 tyrosine kinase inhibitors were only tested in normal mice and not in tumor-bearing mice, indicating that tumor cells were the source of VEGF secretion. In contrast, a study by Ebos *et al*. showed that treatment with sunitinib, a small-molecule inhibitor of VEGF and platelet-derived growth factor tyrosine kinases, resulted in higher VEGF and lower sVEGFR-2 plasma concentrations (44). However, this study was also performed in nontumor-bearing mice and no analysis of sVEGFR-2 plasma concentration was performed on tumor-bearing mice treated with sunitinib.

The corresponding receptor fragment sVEGFR-2 decreased significantly in vehicle control mice in our study, whereas cediranib-treated mice exhibited only a slight decrease. In another study by Ebos *et al*., an inverse relationship between sVEGFR-2 plasma concentration and tumor size in untreated animals was also observed (45). In contrast, increased circulating sVEGFR-2 plasma concentrations have been reported in patients with certain types of cancer (46). We investigated whether sVEGFR-2 may serve as a marker of cediranib activity. Several clinical studies with VEGFR-2 inhibitors have reported marked decreases in sVEGFR-2 plasma concentrations, which are accompanied by increases in VEGF (19, 42, 47, 48). The source of sVEGFR-2 remains unclear. Interestingly, in the study by Ebos *et al*., decreases in sVEGFR-2 in tumor-bearing animals were reported to be mediated largely by tumor-derived VEGF (45). In the first clinical phase I study, cediranib showed encouraging antitumor activity in patients with a broad range of advanced solid tumors (19). Following once-daily dosing of cediranib, time- and dose-dependent reductions in sVEGFR-2 plasma concentrations were demonstrated. Increases in VEGF were detected following dosing acutely even after 1 dose of cediranib, but there was no suggestion of a dose relationship, possibly indicating that even low doses of cediranib that did not show antitumor activity might cause an acute stress response in the tumor. In a study with sunitinib, a multi-targeted tyrosine kinase inhibitor, in patients with metastatic imatinib-refractory gastrointestinal stromal tumors, plasma concentrations of VEGF increased by 2.2-fold and sVEGFR-2 decreased by 25% during the first 2 weeks of treatment (42). These changes were reversed during periods in which patients received no treatment.

In contrast to the clinical studies, cediranib-treated animals in our study exhibited only a slight, but not significant, decrease in sVEGFR-2 plasma concentration. One possible reason could be that the decrease in the tumor burden of cediranib-treated animals was more marked as most patients in clinical trials with antiangiogenic therapies achieved only stable disease. In our study, there was no correlation between absolute sVEGFR-2 plasma concentrations and tumor size in either cediranib-treated or vehicle control animals after short- or long-term treatment. However, when the data were expressed as relative sVEGFR-2 plasma concentration ($\Delta\%$ $sVEGFR\text{-}2_{d9–d21}$), cediranib-treated animals had lower relative sVEGFR-2 plasma concentrations and tumor burdens (p=0.1334) compared with vehicle control animals.

In conclusion, targeting of VEGF signaling with cediranib significantly inhibited angiogenesis and primary tumor growth in this clinically relevant orthotopic RENCA model. These findings indicate that cediranib can cause vascular regression in tumors and this is possibly due to a direct action of cediranib on tumor endothelium, derived from potent inhibition of VEGF signaling and neovascular survival. Changes of VEGF and sVEGFR-2 plasma concentrations may demonstrate biological activity of cediranib.

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