Therapeutics, Targets, and Chemical Biology

Cancer Research

Sunitinib Acts Primarily on Tumor Endothelium rather than Tumor Cells to Inhibit the Growth of Renal Cell Carcinoma

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Abstract

Sunitinib is a broad-spectrum small-molecule inhibitor of receptor tyrosine kinases (RTK) that serves as the present standard of care for first-line therapy of advanced clear cell renal cell carcinoma (ccRCC). A full understanding of the targets and mechanism of action of sunitinib in ccRCC treatment remains incomplete. In this study, we evaluated several tumor cell and endothelial targets of sunitinib and investigated which RTK(s) may specifically contribute to its therapeutic effects. Microarray expression profiling and Western blot analysis revealed that among known sunitinib targets, only platelet-derived growth factor receptor-β and vascular endothelial growth factor receptor-2 (VEGFR-2) were overexpressed in ccRCCs relative to normal tissues. Sunitinib was unable to inhibit survival or proliferation of ccRCC cells at pharmacologically relevant concentrations (~0.1 μmol/L) that inhibit RTK targets. In contrast, sunitinib inhibited endothelial cell proliferation and motility at the same concentrations by suppressing VEGFR-2 signaling. Moreover, whereas sunitinib inhibited the growth of ccRCC xenograft tumors and decreased tumor microvessel density as soon as 12 hours after treatment, sunitinib showed no significant effects on tumor cell proliferation or apoptosis up to 72 hours after treatment. Our findings indicate that sunitinib inhibits ccRCC growth primarily through an antiangiogenic mechanism and not through direct targeting of ccRCC tumor cells. *Cancer Res; 70(3); 1053–62. ©2010 AACR.*

Introduction

Sunitinib is a multitargeted inhibitor of receptor tyrosine kinases (RTK) and has shown clinical efficacy in the treatment of human cancers. It is currently Food and Drug Administration approved for the treatment of advanced clear cell renal cell carcinoma (ccRCC) and gastrointestinal stromal tumors. It is also under intense investigation as a treatment for several other cancers, including breast cancer, colorectal cancer, and non–small cell lung cancer (1–4). Sunitinib has been shown to inhibit the tyrosine kinase activity of vascular endothelial growth factor receptors (VEGFR-1, VEGFR-2, and VEGFR-3), platelet-derived growth factor receptors (PDGFR-α and PDGFR-β), fms-related tyrosine kinase 3 (FLT3), stem cell growth factor receptor KIT, and RET (5).

The ability of sunitinib to suppress tumor angiogenesis has been well established. Two of the major targets of sunitinib, VEGFR and PDGFR, are expressed on endothelial cells and perivascular cells (pericytes) and play critical roles in angiogenesis. Sunitinib may also have direct antiproliferative and/or apoptotic effects on tumor cells, which express target kinases. Sunitinib has been shown to directly inhibit the survival and proliferation of a variety of cancer cells, including small cell lung carcinoma, gastrointestinal stromal tumors, acute myelogenous leukemia, and chronic myelogenous monocytic leukemia, which harbored either activating mutations or translocations of the target RTKs or their ligands (1, 3, 4, 6–8). However, direct antitumor effects of sunitinib in the treatment of ccRCC have not been clearly established. To date, no somatic mutations in target RTK genes have been found in human ccRCC (9). Although one recent study reported apoptotic effects of sunitinib on ccRCC cells *in vitro*, the doses observed to inhibit cell survival were far higher than pharmacologically relevant doses (10). Thus, despite the efficacy of sunitinib in the treatment of ccRCC, its targets and complete mechanism of action against ccRCC remain unclear. In particular, it is unclear whether sunitinib acts primarily through an antiangiogenic mechanism or whether it may also act to directly target ccRCC tumor cells.

Sunitinib is currently considered the standard of care for first-line therapy of metastatic ccRCC, a disease that is resistant to traditional chemotherapy and radiotherapy and that has long had a very poor patient survival rate. Compared with traditional IFN treatment, sunitinib more than doubles progression-free survival of metastatic ccRCC (11, 12). However, not all patients respond to sunitinib, and the vast majority eventually develop resistance to sunitinib therapy. A complete understanding of the mechanisms of sunitinib action against ccRCC is thus critical in understanding and improving

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treatment of this disease. In this study, we evaluated the primary targets of sunitinib in both ccRCC tumor cells and endothelial cells. We determined that, at pharmacologically relevant concentrations, the primary action of sunitinib in ccRCC is through an antiangiogenic mechanism and not through direct targeting of ccRCC cells.

Materials and Methods

Reagents

Sunitinib was provided by Pfizer Global Pharmaceuticals and was prepared as a 20 mmol/L stock solution in DMSO (Sigma) for *in vitro* studies.

Expression of RTKs in Clear Cell RCC

The mRNA expression of RTK-related genes was measured in 174 clear cell renal tumors and 15 normal kidney samples using Affymetrix HGU133 Plus 2.0 microarrays, as described elsewhere (13).

Cells and Cell Culture

ACHN, A-498, and 786-O ccRCC cell lines were obtained from the American Type Culture Collection. SN12C cells were kindly provided by Dr. George Vande Woude (Van Andel Research Institute, originally from National Cancer Institute). The cells were maintained in DMEM or RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) in a humidified incubator containing 5% CO_2 at 37°C. Human umbilical vascular endothelial cells (HUVEC) and human lymphatic microvascular endothelial cells (HLMVEC) were obtained from Clonetics and maintained in Clonetics EBM-2 medium supplemented with EGM-2 SingleQuots or EGM-MV SingleQuots (Lonza). All the cell lines were used within 20 passages.

Immunoprecipitation and Immunoblotting

Cells were starved in low serum (0.1% FBS) overnight before treatment with sunitinib for 2 h. After treatment, cells were stimulated with 50 ng/mL recombinant human PDGF-BB or VEGF165 (R&D Systems) for 10 min. Cells were then lysed with cold HNTG lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, and 1 mmol/L EGTA) containing protease and phosphatase inhibitors [10 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail (Roche Diagnostics)], and protein concentration was determined using the detergent-compatible protein assay according to the manufacturer's instructions (Bio-Rad). Protein (1 mg) from each sample was immunoprecipitated overnight at 4°C with an anti–PDGFR-β (Santa Cruz Biotechnology) or anti–VEGFR-2 (Cell Signaling) antibody and protein G/A agarose beads (Pierce). Immune complexes were washed with cold HNTG lysis buffer containing inhibitors. Proteins were eluted by boiling in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were probed with an anti-phosphotyrosine antibody (Upstate) and then stripped with stripping buffer. To detect total PDGFR-β and VEGFR-2 levels, membranes were reprobed with the same anti–PDGFR-β and anti–VEGFR-2 antibody that was used for the immunoprecipitation. Western blot analysis of phospho–extracellular signal-regulated kinase (ERK) 1/2 (Cell Signaling), ERK1/2 (Cell Signaling), phospho-AKT (Cell Signaling), and AKT (Cell Signaling) was performed on whole-cell lysates (30 μg). β-Actin (Abcam) was used as a loading control.

Ligand-Dependent Cell Proliferation Assay

Cells were plated in 96-well plates at a density of 3,000 per well and starved in low serum overnight in a medium containing 0.1% FBS. Cells were treated with sunitinib and stimulated with 50 ng/mL recombinant human PDGF-BB or PDGF-AA or VEGF165 (R&D Systems) and cultured continuously in the presence of sunitinib for 72 h. Cell viability was assessed using the MTS assay (Promega). MTS assays were also performed on RCC cells growing in complete medium (10% FBS) treated with sunitinib. Results were expressed as a percentage of viable cells relative to cells treated with DMSO. Experiments were performed in triplicate and repeated at least three times.

Small Interfering RNA Transfection

RCC cells were seeded in 96-well or 6-well plates and, 24 h later, transfected with individual small interfering RNA (siRNA; Invitrogen) targeting PDGFR-α, PDGFR-β, and VEGFR-1, respectively, using Oligofectamine (Invitrogen) according to the manufacturer's protocol. At the same time, nontargeting siRNA (Invitrogen) was transfected as a negative control (Neg-L or Neg-H means with low or high GC content). Twenty-four, 48, and 72 h after transfection, cell viability was determined using MTS assay, and the knockdown of individual gene was confirmed by Western blotting 72 h after transfection.

Soft Agar Colony Formation Assay

Soft agar colony formation assay was performed as described elsewhere (14). Cells were treated with either sunitinib (0.1, 1, and 5 μmol/L) or 0.1% DMSO (control). Experiments were performed in triplicate and repeated three times.

Cell Cycle Analysis

Cells were incubated with either 0.1 μmol/L sunitinib or DMSO (control) for 72 h. Cells were collected and analyzed using a cellular DNA flow cytometric analysis kit (Roche Diagnostics). Cell cycle profiles were determined by flow cytometric analysis. Daunorubicin (0.1 μmol/L) was used as a positive control for cell cycle arrest at G_2-M phase.

Endothelial Cell Invasion Assay

Endothelial cell invasion was analyzed using the BD BioCoat endothelial cell invasion system according to the instructions of the manufacturer (BD Biosciences). Briefly, HUVEC cells (2×10^4) were plated onto the upper well of the chamber with medium (serum-free) alone with or without sunitinib; the lower chamber contained 50 ng/mL VEGF

(R&D Systems) as a chemoattractant. All experimental samples were set up in duplicate. Cells were incubated at 37°C in CO_2 for 16 to 24 h, and then residual cells on the upper surface of the chamber membranes were removed using a cotton swab. Invaded cells on the lower membrane surface were visualized by Diff-Quik staining kit (Allegiance) according to the manufacturer's protocol and examined under the microscope. Pictures of invaded cells were taken for three fields per well under ×100 magnification, and the number of invaded cells was counted by ImageJ v1.37v software.

Xenograft Models

Tumor implantation and growth. All animal studies were in compliance with Van Andel Research Institute Institutional Animal Care and Use Committee policies. Six-week-old female BALB/c *nu/nu* nude mice (Charles River) were given s.c. injections of 3×10^6 A-498, ACHN, SN12C, or 786-O cells in the right flank. Tumor size was measured twice per week using digital calipers (Mitutoyo), and tumor volume was calculated as length × width × height × 0.5. Tumor growth ratio is presented as mean + SD and normalized to the initial volume when sunitinib treatment began. When tumors had grown to an average volume of 200 to 300 mm^3, tumor-bearing mice were separated into four groups of 10 animals (two groups for A-498 and 786-O xenografts). Three treatment groups received an oral gavage of sunitinib as a citrate-buffered (pH 3.5) solution once daily at the dosages of 20, 40, and 80 mg/kg (40 mg/kg for A-498 and 786-O xenografts), respectively. At the same time, one vehicle control group received citrate buffer (pH 3.5) only. Mice were euthanized at the end of the treatment period. Tumors were removed and fixed in 4% paraformaldehyde and paraffin embedded, and then 4-μm-thick sections were prepared. Some sections were stained with H&E and the others were used for subsequent immunohistochemical analysis.

Target modulation study. To determine the mechanism of action of sunitinib in these ccRCC xenograft models, a separate study was designed in which sunitinib treatment was initiated when tumors grew untreated to a size of 400 to 500 mm^3. Mice were then administered sunitinib or vehicle control at the indicated concentration once daily for 1 to 4 d. At the indicated time after dosing, individual mice were sacrificed and tumors were resected and fixed in 4% paraformaldehyde. Microvessel density (MVD) was determined by CD34 staining in the control- or sunitinib-treated tumor sections. Tumor cell proliferation and apoptosis were also determined by proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining as described below.

Immunohistochemistry

Immunohistochemical staining for PCNA, TUNEL, and CD34 was performed as described elsewhere (14). PCNA antibody (Abcam) was used at a dilution of 1:500. TUNEL staining was performed according to the protocol of the manufacturer (Promega). CD34 (MEC 14.7, Abcam) antibody was used at a dilution of 1:50. The quantification of MVD, proliferation index, and apoptosis index was also performed as described elsewhere (14).

Statistical Analysis

All values are expressed as mean ± SD. Values were compared using Student's t test. $P < 0.05$ was considered significant.

Results

PDGFR-β and VEGFR-2 are highly expressed in human ccRCC. To identify potential RTK(s) that would likely be the targets of sunitinib in ccRCC, we examined the expression levels of several RTKs, including VEGFR, PDGFR, FLT3, c-KIT, and RET, in 174 human ccRCC samples as determined by gene expression profiling. We found that PDGFR-β and VEGFR-2, but not others, were highly expressed in human ccRCC when compared with normal controls ($P < 0.001$; Fig. 1A). We also examined the expression and activation of these RTKs in ccRCC cell lines and endothelial cell lines by Western blotting. PDGFR-β was expressed by 4 of 12 RCC cell lines, PDGFR-α was expressed by most RCC cell lines, VEGFR-1 was expressed in almost all RCC and endothelial cell lines, whereas VEGFR-2 was expressed only in the endothelial cell lines. However, no significant basal activation of these RTKs was detected either in the ccRCC cells or in the endothelial cells (Fig. 1B; data not shown). FLT3, c-KIT, and RET expression were undetectable in all cell lines. Based on the combination of these data, we focused our study on PDGFR and VEGFR signaling in these respective cells.

***Sunitinib inhibits RTK signaling pathways in ccRCC cells* in vitro.** Pharmacokinetic and pharmacodynamic studies have revealed that the pharmacologically relevant concentration for sunitinib is 50 to 100 ng/mL (0.125–0.25 μmol/L; refs. 4, 15). To examine the effect of sunitinib at these concentrations on the activation of PDGFR-β in ccRCC cells, two PDGFR-β–expressing cell lines, SN12C and ACHN, were chosen for evaluation *in vitro*. Sunitinib decreased PDGF-BB–stimulated phosphorylation of PDGFR-β at 0.01 μmol/L in both cell lines and completely inhibited the phosphorylation of PDGFR-β at concentrations ≥0.1 μmol/L (Fig. 1C).

RTKs transduce signals of proliferation, migration, and survival from the extracellular environment through a series of downstream signal transduction pathways. The ERK and AKT kinases are two major downstream effectors of PDGFR signaling (16, 17). Western blot analysis showed that, in comparison with DMSO controls, sunitinib inhibited PDGF-BB–stimulated activation of ERK and AKT at ~0.1 μmol/L (Fig. 1D), the same concentration shown to completely inhibit activation of PDGFR-β. Because sunitinib shows similar inhibitory potency (K_i value) on both PDGFR and VEGFR in biochemical assays (5), we speculate that sunitinib could inhibit the activation of these RTKs simultaneously at the above concentrations.

Proliferation of ccRCC cells is not affected by sunitinib at concentrations that inhibit RTK signaling. To further explore the role of PDGFR and VEGFR signaling pathways in

ccRCC cells *in vitro*, cancer cells were serum starved and then PDGFR and VEGFR signaling pathways were activated with PDGF and VEGF. The IC_{50} for sunitinib to inhibit PDGF- or VEGF-dependent proliferation of ccRCC cells was 4 to 10 μmol/L, which is much higher than the concentration of sunitinib sufficient to inhibit RTK phosphorylation (0.01–0.1 μmol/L). Sunitinib showed similar effects on proliferation of ccRCC cells in PDGF- or VEGF-only medium and in complete medium (Fig. 2A; Supplementary Fig. S1A). This indicates that inhibition of the PDGFR and VEGFR signaling pathways is not sufficient to inhibit the proliferation of ccRCC cells *in vitro*. To our surprise, PDGF or VEGF alone did not induce the proliferation of ccRCC cells after serum starvation (Fig. 2B; Supplementary Fig. S1B), although PDGF has been reported to stimulate the proliferation of PDGFR-transfected NIH-3T3 cells (4). Cell cycle analysis also showed no change in cell cycle progression after sunitinib treatment compared with DMSO controls (Supplementary Fig. S2).

Knockdown of PDGFR and VEGFR does not affect proliferation of ccRCC cells. Based on the above findings, we hypothesized that the effect of sunitinib on ccRCC is not attributable to the direct targeting of ccRCC cells. To test this hypothesis, we further examined the effect of knockdown of PDGFR and VEGFR on the proliferation of ccRCC cells using siRNA *in vitro*. Knockdown of individual PDGFR or VEGFR isoforms did not affect the proliferation of ccRCC cells compared with the nontargeting siRNA controls (negative; Fig. 2C; Supplementary Fig. S3).

Sunitinib inhibits anchorage-independent growth of ccRCC cells at higher concentrations. Because anchorage-independent growth is a characteristic of transformed cells, we next used a soft agar colony formation assay to evaluate whether sunitinib could reverse malignant properties of ccRCC cells at pharmacologically relevant concentrations. Although sunitinib significantly inhibited the anchorage-independent colony formation of ACHN and A-498 cells at 5 μmol/L ($P < 0.01$), it showed weak effects at 1 μmol/L and no effect at 0.1 μmol/L (Fig. 3; Supplementary Fig. S4).

***Sunitinib inhibits VEGFR-2–mediated endothelial cell growth and invasion* in vitro.** Because VEGFR has been found important for tumor angiogenesis and is expressed in tumor-associated endothelial cells, we next investigated

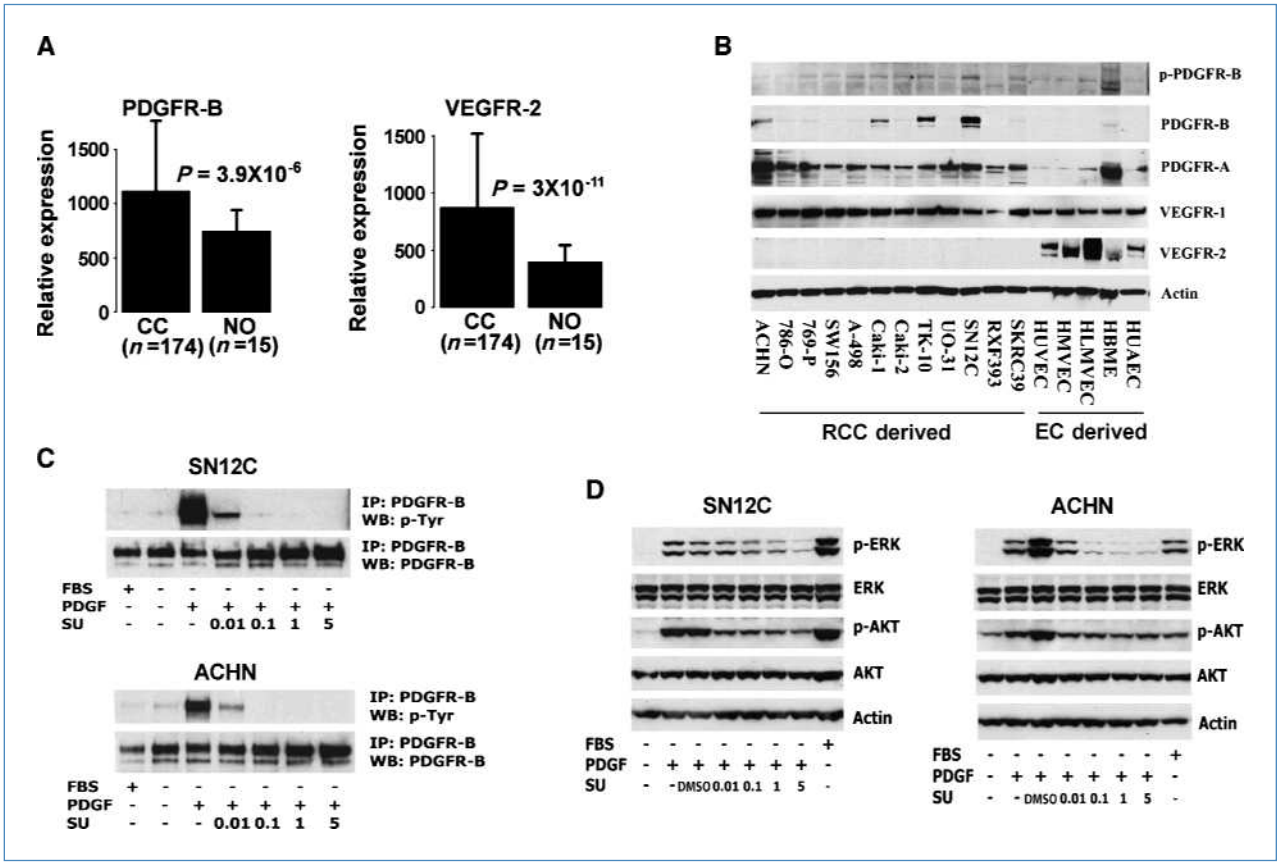

Figure 1. Expression profile of RTKs and the effect of sunitinib on RTK signaling in ccRCC cells. A, overexpression of PDGFR-β and VEGFR-2 in 174 human clear cell RCC samples (*CC*) and 15 normal controls (*NO*) is shown by Affymetrix microarray analysis. Columns, average gene expression level values of each group; bars, SD. B, expression of PDGFR and VEGFR in ccRCC cell lines and endothelial cell lines is shown by Western blots. C, phosphorylation of PDGFR-β was inhibited by sunitinib (*SU*) at pharmacologically relevant concentrations in SN12C and ACHN cells. D, sunitinib inhibited PDGF-BB–stimulated activation of ERK and AKT signaling in comparison with DMSO controls at about 0.01 to 0.1 μmol/L, the same concentrations that inhibited activation of PDGFR-β.

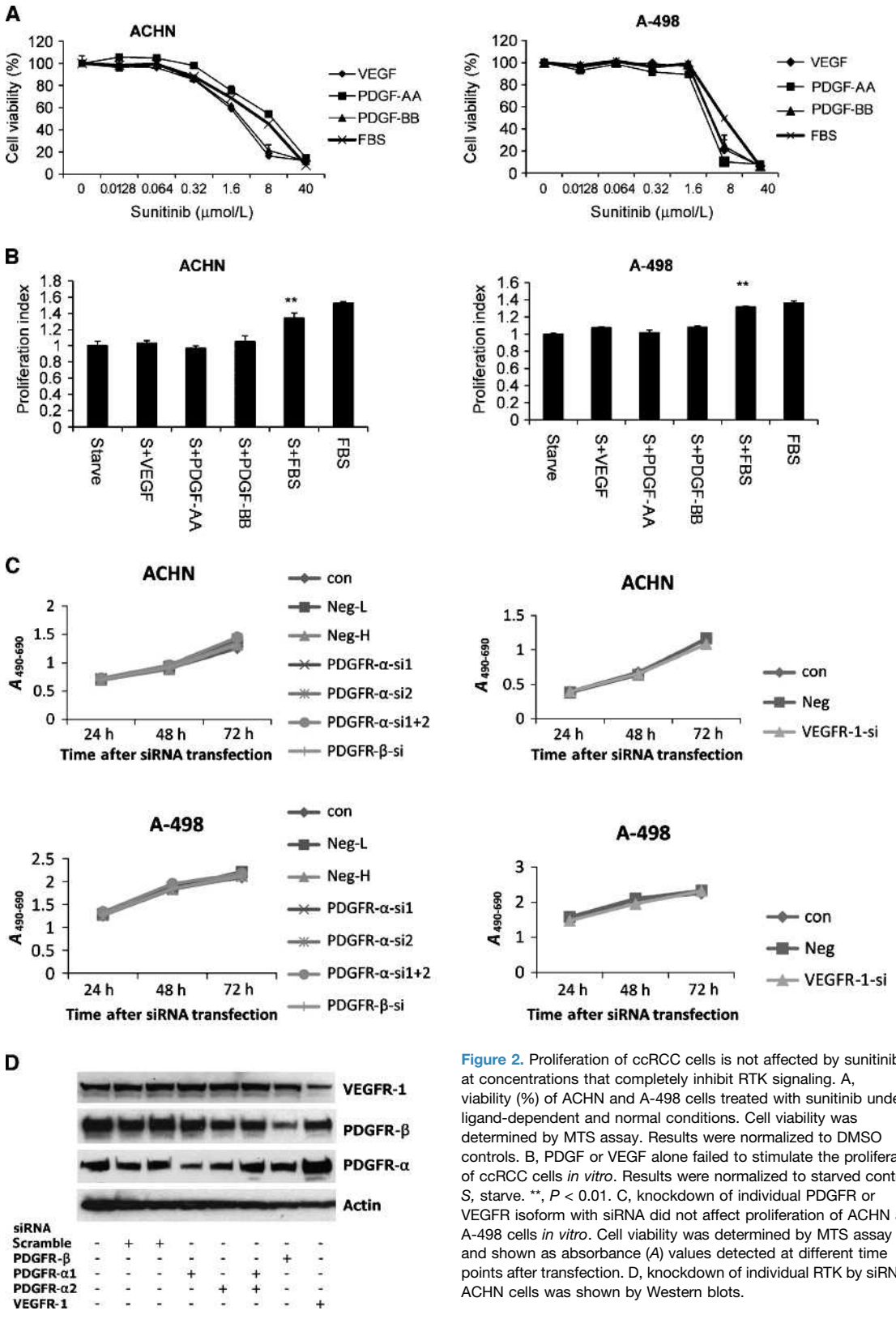

Figure 2. Proliferation of ccRCC cells is not affected by sunitinib at concentrations that completely inhibit RTK signaling. A, viability (%) of ACHN and A-498 cells treated with sunitinib under ligand-dependent and normal conditions. Cell viability was determined by MTS assay. Results were normalized to DMSO controls. B, PDGF or VEGF alone failed to stimulate the proliferation of ccRCC cells *in vitro*. Results were normalized to starved controls. *S*, starve. **, $P < 0.01$. C, knockdown of individual PDGFR or VEGFR isoform with siRNA did not affect proliferation of ACHN and A-498 cells *in vitro*. Cell viability was determined by MTS assay and shown as absorbance (*A*) values detected at different time points after transfection. D, knockdown of individual RTK by siRNA in ACHN cells was shown by Western blots.

the effect of sunitinib on endothelial cell lines *in vitro*. First, we examined the expression of the RTKs in five human endothelial cell lines. VEGFR-1 and VEGFR-2 were highly expressed in those cell lines; in contrast, PDGFR expression was weak or undetectable (Fig. 1B). Analogous to its effect on PDGFR activation in ccRCC cell lines, sunitinib could inhibit the phosphorylation of VEGFR in HLMVEC cells at concentrations between 0.01 and 0.1 μmol/L (Fig. 4A). In addition, ERK signaling downstream of VEGFR was also inhibited by sunitinib at this concentration [VEGFR-mediated activation of AKT (Ser^{473}) was undetectable in our system]. Compared with ccRCC tumor cells, sunitinib showed more robust inhibitory effect on the proliferation of HUVEC and HLMVEC endothelial cells. IC_{50} values on endothelial cell lines under VEGF-dependent conditions were ~0.01 μmol/L, which was similar to the concentrations required to inhibit activation of VEGFR-2 (Fig. 4B). We further found that after serum starvation, VEGF alone could induce the proliferation of endothelial cells (Fig. 4C). At pharmacologically relevant concentration, sunitinib also inhibited VEGF-induced endothelial cell invasion (Fig. 4D). These results show that VEGFR signaling is required for proliferation and invasion of endothelial cells and that inhibition of VEGFR signaling with sunitinib suppresses the function of endothelial cells. Thus, all the above results indicate that at pharmacologically relevant concentrations, sunitinib may primarily target tumor endothelium rather than ccRCC cells *in vivo*.

***Sunitinib inhibits RCC xenograft tumor growth and tumor angiogenesis* in vivo.** To investigate the effect of sunitinib *in vivo* and the effect of *VHL* status on response to sunitinib, mice bearing tumor xenografts of ccRCC cell lines were treated with sunitinib. Four ccRCC cell lines were chosen for xenograft study: A-498 and 786-O cells contain loss of heterozygosity *VHL*, whereas ACHN and SN12C cells harbor wild-type *VHL*. Sunitinib treatment showed either growth inhibition (40 mg/kg) or stasis (80 mg/kg) effects on SN12C xenografts (data not shown), whereas sunitinib treatment caused stasis effects at 40 mg/kg on A-498 and 786-O xenografts and induced growth inhibition at 20 mg/kg and regression at 40 and 80 mg/kg for ACHN xenografts (Fig. 5A; Supplementary Fig. S5A). Quantification of tumor MVD at the end of sunitinib treatment showed that MVD was significantly decreased in sunitinib-treated tumors compared with vehicle controls (Fig. 5B; Supplementary Fig. S5B).

***Sunitinib primarily inhibits ccRCC tumor angiogenesis but not ccRCC cells* in vivo.** To further elucidate the mechanism of sunitinib-mediated growth inhibition or stasis effect *in vivo*, we collected tumor samples treated with sunitinib for 24 to 96 hours and examined the effect of sunitinib on tumor vasculature as well as ccRCC cell proliferation and apoptosis. In A-498 xenografts, tumor MVD was significantly inhibited by sunitinib as soon as 12 hours after treatment compared with vehicle-treated controls (Fig. 6A; also see results for SN12C xenografts in Supplementary Fig. S6A). The percentage of PCNA-positive cells (shown as proliferation index) and the percentage of TUNEL-positive cells (shown as apoptosis index) in the viable tumor section did not significantly alter up to 72 hours after sunitinib treatment when compared with vehicle controls (Fig. 6B; also see results for SN12C xenografts in Supplementary Fig. S6B). This result suggests that the inhibitory effect of sunitinib on xenograft tumor growth *in vivo* was not caused by direct targeting of ccRCC cells. The

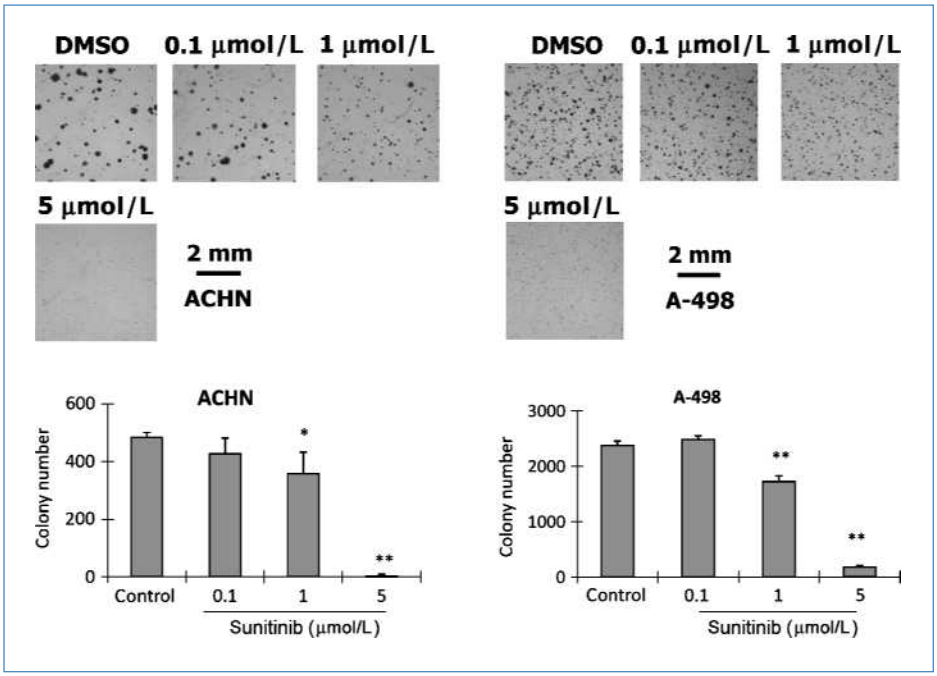

Figure 3. Sunitinib does not affect the anchorage-independent growth of ccRCC cells at pharmacologically relevant concentrations. Cells were treated with either sunitinib (0.1, 1, and 5 μmol/L) or 0.1% DMSO (control). Although sunitinib significantly inhibited the anchorage-independent colony formation of ACHN and A-498 cells at 5 μmol/L, it showed weak effects at 1 μmol/L and no effect at 0.1 μmol/L. *, $P < 0.05$; **, $P < 0.01$.

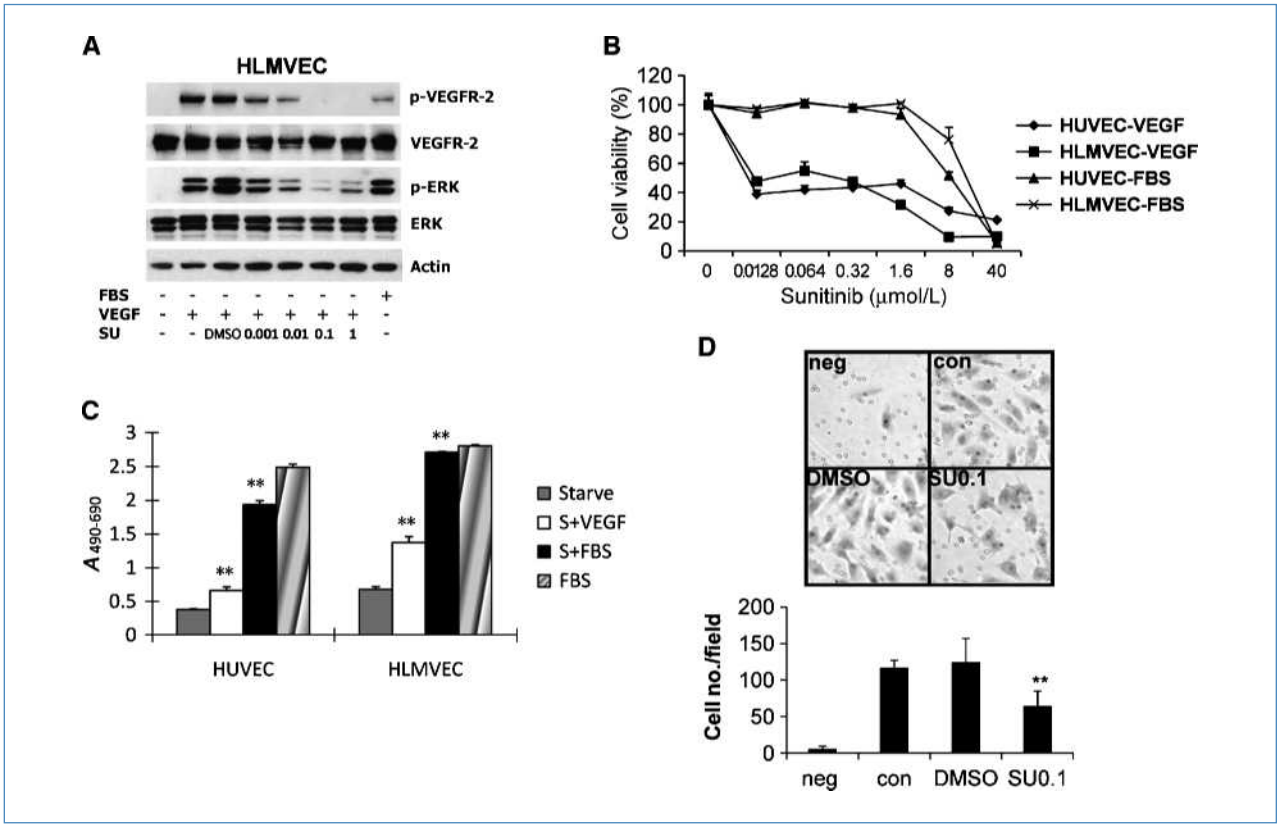

Figure 4. Sunitinib inhibits phosphorylation of VEGFR-2 and VEGF-dependent proliferation and invasion of endothelial cells at pharmacologically relevant concentrations. A, sunitinib inhibited the phosphorylation of VEGFR-2 and downstream ERK signaling in HLMVEC endothelial cells at pharmacologically relevant concentrations (0.01–0.1 μmol/L). B, sunitinib inhibited VEGF-dependent proliferation of endothelial cells as analyzed by MTS assay. Results were normalized to DMSO controls. C, VEGF induced the proliferation of endothelial cells *in vitro*. **, $P < 0.01$. D, sunitinib inhibited VEGF-dependent invasion of endothelial cells at pharmacologically relevant concentrations. VEGF was used as a chemoattractant for control (*con*)–treated, DMSO-treated, or sunitinib (0.1 μmol/L)–treated samples; negative (*neg*) means no chemoattractant added. **, $P < 0.01$.

inhibition of endothelial cell function by sunitinib *in vitro* and the reduction of MVD in the sunitinib-treated tumors *in vivo* indicate that the antitumor effect of sunitinib is mainly mediated by inhibition of tumor angiogenesis.

Discussion

Sunitinib is currently considered first-line therapy for the treatment of advanced ccRCC. However, the relationship between clinical efficacy of sunitinib on ccRCC and its mechanism of action has not been established. To study this problem, we directly examined the effect and action of sunitinib on ccRCC cells and endothelial cells *in vitro* and *in vivo* at pharmacologically relevant concentrations. Our studies indicate that sunitinib inhibits ccRCC growth primarily through an antiangiogenic mechanism and not through direct targeting of ccRCC tumor cells.

A recent study by Xin and colleagues (10) reported that sunitinib induces apoptosis in ccRCC cells through inhibition of signal transducer and activator of transcription 3. However, Xin and colleagues found that the IC_{50} values required to inhibit ccRCC cell viability and proliferation *in vitro* were in the range of 5 μmol/L and above, >50 times the concentration of sunitinib required to inhibit RTK signaling in our cell lines (0.01–0.1 μmol/L; see Fig. 1). Other groups have also reported that sunitinib concentrations much lower than 5 μmol/L are sufficient to inhibit RTK signaling. In cellular assays, Mendel and colleagues (4) found that sunitinib inhibited ligand-dependent phosphorylation of VEGFR-2 and PDGFR-β with IC_{50} values of ~0.01 μmol/L, consistent with our own findings. Finally, both clinical and preclinical pharmacodynamic and pharmacokinetic studies indicate that pharmacologically and clinically relevant plasma concentrations of sunitinib are in the range of 50 to 100 ng/mL or 0.1 to 0.2 μmol/L (4, 15). Thus, we believe that the high concentrations of sunitinib (~5 μmol/L), which Xin and colleagues observed to inhibit proliferation and viability of ccRCC cells, are likely not pharmacologically or clinically relevant.

In our study, we observed that pharmacologically relevant concentrations of sunitinib (~0.1 μmol/L) did not affect the viability or proliferation of ccRCC cell lines *in vitro*. In contrast, sunitinib did inhibit endothelial cell proliferation and invasion and did so at concentrations that also inhibited VEGFR signaling in these cells. Moreover, studies of ccRCC xenografts treated with pharmacologically relevant concentrations of sunitinib showed that sunitinib rapidly inhibits

tumor angiogenesis *in vivo* but did not affect the proliferation or apoptosis of ccRCC tumor cells. Together, these data indicate that sunitinib acts primarily to target tumor vasculature rather than tumor cells in the treatment of ccRCC.

Our analysis of RTK expression in ccRCC suggested that PDGFR and VEGFR were likely relevant targets of sunitinib in the treatment of ccRCC. PDGFR-β and VEGFR-2 were both overexpressed in human ccRCC tumor samples compared with normal tissue. Analysis of ccRCC and endothelial cell lines found that, of the known sunitinib-sensitive RTKs, only PDGFR-α, PDGFR-β, and VEGFR-1 were expressed by ccRCC cells, whereas VEGFR-1 and VEGFR-2 were highly expressed by endothelial cells. Overexpression of c-KIT has been shown in chromophobe RCC and renal oncocytoma but not ccRCC (18, 19). Although higher PDGFR-α expression has been correlated with RCC progression (20, 21), our *in vitro* studies showed that inhibition of PDGFR signaling (by either sunitinib treatment or siRNA knockdown of PDGFR expression) was not sufficient to inhibit the proliferation of ccRCC cells. Knockdown of VEGFR-1 expression in ccRCC cells also failed to inhibit proliferation. Thus, PDGFR and VEGFR-1 signaling in ccRCC cells are likely redundant growth signaling pathways. In contrast, we found that VEGFR signaling is required for the proliferation of endothelial cells. Sunitinib inhibited phosphorylation of VEGFR- and VEGF-dependent proliferation of endothelial cells at similar concentrations (0.01 μmol/L), indicating the important role of VEGFR signaling for proliferation of endothelial cells.

Although PDGFR signaling may not be required for growth of ccRCC cells, it has been shown as a requirement for the survival of pericytes, perivascular cells surrounding newly formed blood vessels, in several cancer types (22–24). In the neovascularization process, endothelial cells secrete growth factors that recruit pericytes to the newly formed blood vessel to stabilize and mature the vascular network. PDGF-B is expressed by sprouting capillary endothelial cells, whereas its receptor

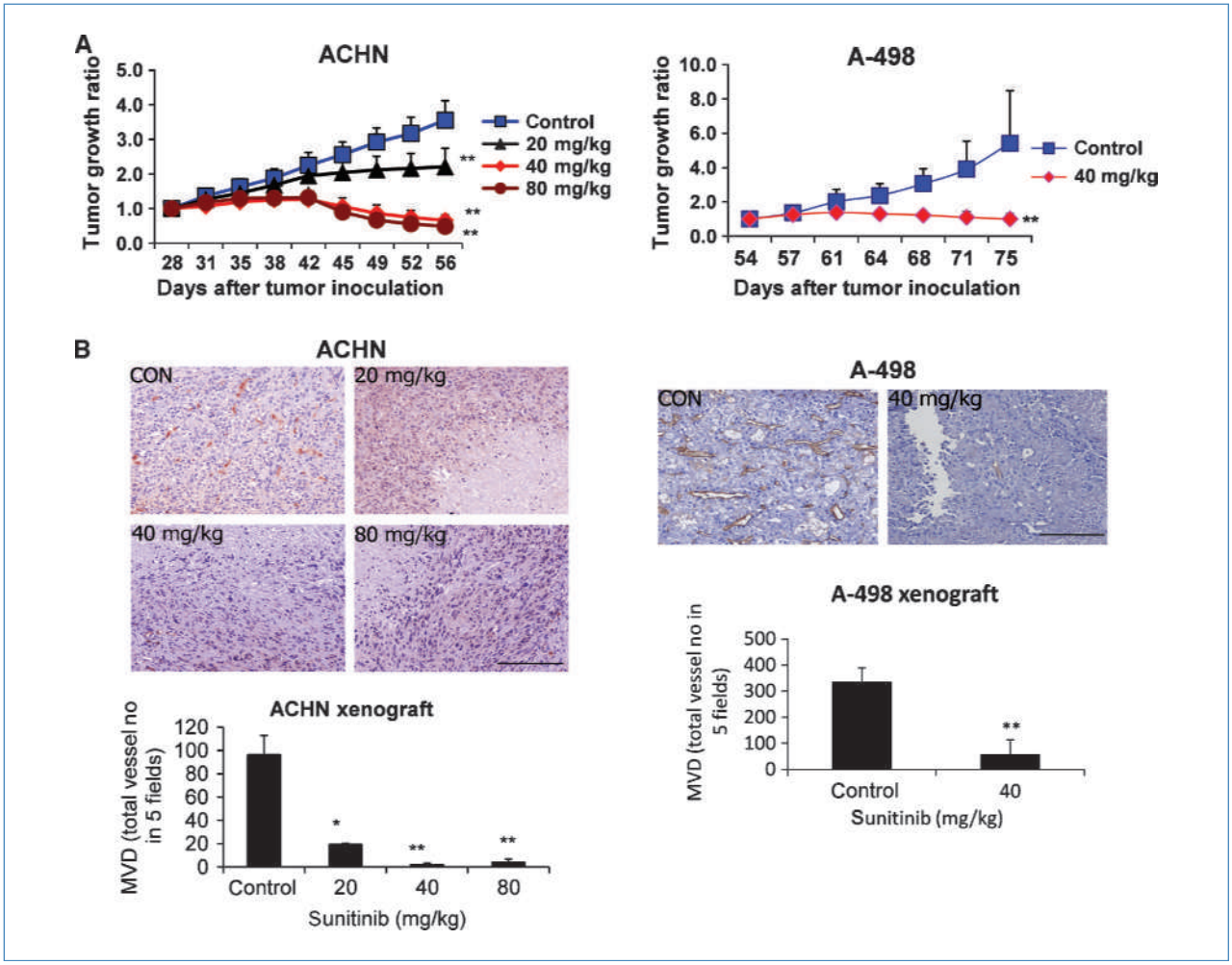

Figure 5. Sunitinib inhibits ccRCC xenograft tumor growth and tumor angiogenesis *in vivo*. A, growth of ACHN and A-498 xenograft tumors was inhibited by sunitinib. **, $P < 0.01$. B, decreased MVD was found in sunitinib-treated tumor sections as evaluated by CD34 staining. *Scale bar,* 0.20 mm. *CON,* control. *, $P < 0.05$; **, $P < 0.01$.

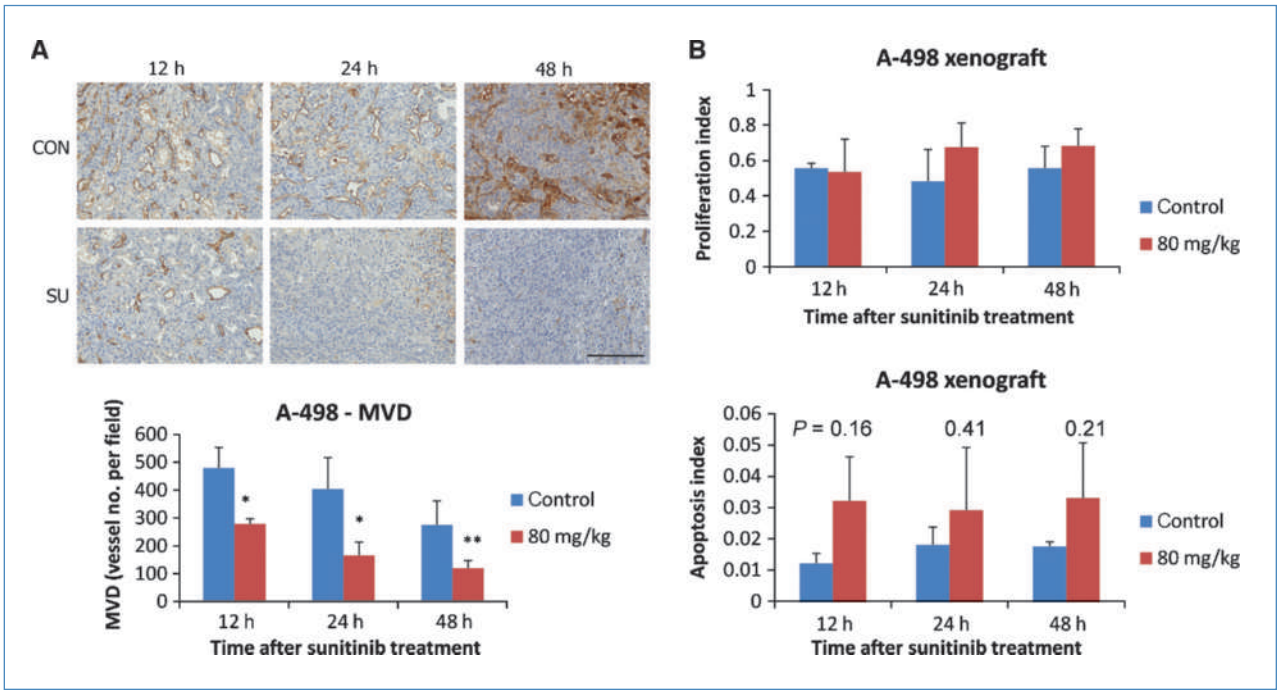

Figure 6. Target modulation study shows that sunitinib primarily acts on tumor endothelium rather than via direct targeting of ccRCC cells *in vivo*. A, sunitinib inhibited A-498 xenograft tumor angiogenesis as soon as 12 h after treatment. *, $P < 0.05$; **, $P < 0.01$. B, sunitinib showed minimal effect on A-498 tumor cell proliferation and did not induce apoptosis of A-498 cells up to 72 h after treatment *in vivo*.

PDGFR-β is localized on pericytes, suggesting a paracrine signaling circuit between the two cell types (25–27). Although sunitinib does not affect the proliferation of ccRCC cells, our preliminary results indicate that it might inhibit pericytes survival through PDGFR suppression (Supplementary Fig. S7) and contribute to the inhibition of tumor angiogenesis. Further studies are needed to validate this hypothesis.

Mutations in the *VHL* gene are associated with the majority of ccRCC tumors (28). We did not see a correlation between *VHL* mutational status and response to sunitinib. We assessed the effect of sunitinib on four ccRCC xenografts (two with $VHL^{-/-}$ and two with $VHL^{+/+}$). The most sensitive xenograft was the $VHL^{+/+}$ ACHN (40 mg/kg sunitinib causes regression). The mid-sensitive xenografts were A-498 and 786-O ($VHL^{-/-}$; 40 mg/kg sunitinib causes stasis effect). The least sensitive xenograft was SN12C ($VHL^{+/+}$; 40 mg/kg sunitinib causes growth inhibition). The lack of correlation between *VHL* mutational status and response to sunitinib therapy is consistent with clinical observations. In a retrospective analysis of ccRCC patients, Choueiri and colleagues (29) found that *VHL* mutation status had little effect on patient response to sunitinib therapy; response rates of *VHL*-mutated and *VHL* wild-type ccRCC patients to sunitinib therapy were 56% and 52%, respectively.

In summary, we found that, under pharmacologically relevant concentrations, sunitinib targets the ccRCC tumor endothelium and not ccRCC cells directly. To our knowledge, this is the first study to clearly show that the clinical efficacy of sunitinib on ccRCC is mediated primarily through an antiangiogenic effect and also not through direct targeting of ccRCC cells. This understanding of sunitinib action should have important implications for the improved treatment of ccRCC.

Disclosure of Potential Conflicts of Interest

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