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Targeting both HIF-1 and HIF-2 in human colon cancer cells improves tumor response to sunitinib treatment

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Abstract

Sunitinib is an oral small-molecule multitargeted receptor tyrosine kinase inhibitor that has recently been shown to have clinical benefit as a single agent in renal cell cancer and gastrointestinal stromal tumors, leading to its Food and Drug Administration approval for treatment of these cancers. However, the benefit is short-lived; and for the majority of cancers, sunitinib single-agent clinical activity is low. Therefore, combination strategies with sunitinib are currently in clinical development. The hypoxia-inducible transcription factors, HIF-1 and HIF-2, induce gene programs important for cancer cell growth and angiogenesis. We hypothesized that inhibiting HIF-1 and HIF-2 would further improve tumor response to sunitinib therapy. To test this hypothesis, *HIF-1α* and *HIF-2α* genes were disrupted in colon cancer cells. We found that disruption of *HIF-1α*, *HIF-2α*, or both *HIF-1α* and *HIF-2α* genes led to improved tumor response to sunitinib. For xenografts in which both *HIF-1α* and *HIF-2α* genes were disrupted, there was prolonged complete remission with sunitinib treatment in 50% of mice. This enhanced response was mediated by two potential mechanisms. First, tumor angiogenesis and perfusion were almost completely inhibited by sunitinib when both *HIF-1α* and *HIF-2α* genes were disrupted. The enhanced inhibitory effect on tumor angiogenesis was mediated by the inhibition of multiple proangiogenic factors, including vascular endothelial growth factor and angiopoietin-like protein 4, and the induction of the antiangiogenic factor, thrombospondin 1. Second, disruption of *HIF-1α*, *HIF-2α*, or both *HIF-1α* and *HIF-2α* genes directly inhibited tumor cell proliferation. These preclinical findings have clinical implications and suggest novel clinical trials. [Mol Cancer Ther 2009;8(5):1148–56]

Introduction

Sunitinib is a multitargeted receptor tyrosine kinase inhibitor that exhibits inhibitory activity against multiple targets including c-KIT, vascular endothelial growth factor receptors (VEGFR; VEGFR1, VEGFR2, and VEGFR3), platelet-derived growth factor receptors (α and β), FLT3, CSF-1R, and RET (1–7). Inhibition of these receptor tyrosine kinases blocks transduction of signals important for tumor growth, survival, and angiogenesis. Phase II and III clinical trials with sunitinib have shown clinical benefit, with significantly improved time to progression in patients with imatinib-resistant gastrointestinal stromal tumors and advanced renal cell cancer (6–9). However, this clinical response to sunitinib is heterogeneous. In phase II clinical trials in patients with other solid tumors, including colorectal cancer, breast cancer, non-small cell lung cancer, hepatocellular carcinoma, and neuroendocrine tumors, sunitinib has shown detectable but low objective response rates (10–15). To improve on sunitinib efficacy, combination therapies with cytotoxic chemotherapy, radiation therapy, and targeted agents are currently under active clinical investigation.

It is likely that resistance to sunitinib treatment develops due to the existence of compensatory pathways in both tumor blood vessels and tumor epithelial cells. At the tumor blood vessel level, preclinical and clinical studies have shown that treatment with antiangiogenic or antivascular agents leads to the compensatory induction of proangiogenic factors including VEGF, placental growth factor, interleukin-8, and basic fibroblast growth factor (16–19). These mechanisms may be responsible for tumor blood vessel resistance to sunitinib inhibition of VEGFRs and platelet-derived growth factor receptors. At the tumor epithelial cell level, sunitinib inhibition of receptor tyrosine kinase phosphorylation decreases survival signaling through AKT and extracellular signal-regulated kinase and decreases glucose uptake (20–23). However, inhibition of tumor cell growth is not long-lasting as shown by clinical trials using fluorodeoxyglucose positron emission tomography imaging. In these studies, sunitinib treatment incompletely inhibited fluorodeoxyglucose uptake or induced rebound fluorodeoxyglucose uptake during the initial post-treatment rest period (22, 23).

Overexpression of the hypoxia-inducible transcription factors, HIF-1α or HIF-2α has been strongly associated with cancer progression (24–29). HIF-1 is composed of the HIF-1α and HIF-1β subunits, and HIF-2 is composed of the HIF-2α and HIF-1β subunits. Whereas HIF-1β

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Note: K. Burkitt and S.Y. Chun contributed equally to this work.

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is constitutively expressed, HIF-1α and HIF-2α protein stability and synthesis are regulated by intratumoral hypoxia, growth factors, and genetic alterations (30–38). Induction of HIF-1 and HIF-2 transactivate gene programs important for inducing tumor angiogenesis, cancer cell growth and survival, and energy metabolism (39–42). As such, tumor induction of HIF-1 and HIF-2 may be a mechanism of escape from the effects of sunitinib therapy.

We hypothesized that inhibition of HIF-1 and HIF-2 in cancer cells would improve response to sunitinib treatment. To test this hypothesis, we generated isogenic human colon cancer cell lines with specific genetic disruption of *HIF-1α*, *HIF-2α*, or both *HIF-1α* and *HIF-2α (HIF-α*s). The effects of sunitinib treatment, both *in vitro* and *in vivo*, were compared in parental and knockout cell lines.

Materials and Methods

Cell Lines

All cancer cell lines were acquired from the American Type Culture Collection. HCT116, LOVO, and HCT15 are human colon cancer cell lines. Isogenic cell lines with somatic disruption of *HIF-1α*, *HIF-2α* and *VEGF* genes were derived from HCT116 (HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}}$, HCT116$^{HIF\text{-}2\alpha\text{-}/\text{-}}$, HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}HIF\text{-}2\alpha\text{-}/\text{-}}$, and HCT116$^{VEGF\text{-}/\text{-}}$), LOVO (LOVO$^{HIF\text{-}1\alpha\text{-}/\text{-}}$), and HCT15 (HCT15$^{HIF\text{-}1\alpha\text{-}/\text{-}}$) cells as described previously (43). Cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen).

Cell Proliferation and Clonogenic Survival Assay

Cells were trypsinized, counted, and plated. For cell proliferation studies, cells were harvested daily for 7 days and stained with trypan blue, and viable (nonblue) cells were counted on a hemocytometer. Student's paired *t* test was used to determine statistical significance between groups. For analysis of colony formation, cells were allowed to grow undisturbed at 37°C in 5% CO_2 for 10 days and then stained with crystal violet.

Xenograft Models

Six-week-old female athymic *nu/nu* mice (Harlan) were implanted with 20×10^6 cells by s.c. injection into the flanks as described previously (43). Tumor sizes in two dimensions were measured with calipers, and volumes were calculated with the formula: $(L \times W^2) \times 0.5$, where L is length and W is width. Student's paired *t* test was used to determine statistical significance between groups. Mice were housed in barrier environments, with food and water provided *ad libitum* as approved by the University of Michigan Animal Care and Use Committee. Xenografts were harvested for subsequent analyses when they reached ~0.5 cm^3.

Sunitinib Treatment

For sunitinib treatment, implanted tumors were allowed to grow for 2 to 4 days, and the mice were treated daily by oral gavage with sunitinib malate (Pfizer) at 40 mg/kg/d for 21 days.

Tumor Angiogenesis

Harvested xenografts were fixed in Tissue-Tek OTC compound (Sakura Finetek) and stored at −80°C. Frozen sections, 10 μm in thickness, were prepared with a Leica Microsystems cryostat. For tumor angiogenesis, microvessel density was determined by staining sections with a monoclonal antibody against the endothelial cell-specific marker CD31 (BD Pharmingen) followed by a biotinylated secondary antibody (Jackson ImmunoResearch Laboratories) and rhodamine-streptavidin (Vector Labs). At least three sections from each of three tumors were examined under fluorescence microscopy. Results are illustrated and quantified in Fig. 3 using Image-Pro Plus software (Media Cybernetics).

Tumor Microvessel Perfusion

Mice bearing tumors were injected i.v. with Hoechst 33342 (40 mg/kg) 2 min before sacrifice. Tumors were fixed in Tissue-Tek OTC compound and stored at −80°C. Frozen sections, 10 μm in thickness, were prepared with a Leica Microsystems cryostat and examined under fluorescence microscopy. At least three sections from each of three tumors were examined under fluorescence microscopy. Results are illustrated and quantified in Fig. 3 using Image-Pro Plus software (Media Cybernetics).

Gene Expression Profiling

HCT116 and HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}HIF\text{-}2\alpha\text{-}/\text{-}}$ xenografts were harvested at ~0.4 cm^3 and total RNA was extracted. Gene expression analyses on the samples were done at the University of Michigan Comprehensive Cancer Center Affymetrix Core Facility. Commercial high-density oligonucleotide arrays were used (GeneChip Human Genome U133A; Affymetrix) following protocols and methods developed by the supplier.

Real-time Reverse Transcription-PCR Analyses

Total RNA from xenografts were extracted, treated with DNase I, and reverse-transcribed. Real-time PCRs were done in triplicate on reverse transcription-derived cDNA, and relative values were calculated. Student's paired *t* test was used to determine statistical significance between groups.

Western Blot Analyses

Whole-cell protein extracts were prepared from cell lines, separated by electrophoresis, transferred to nitrocellulose membranes, and probed with antibodies. Antibodies were obtained from Cell Signaling Technologies (peroxidase-conjugated anti-rabbit antibody and peroxidase-conjugated anti-mouse antibody), BD Transduction Laboratories (anti-human HIF-1α), Novus Biologicals (anti-human HIF-2α), and Sigma (anti-α-tubulin). Antibody dilutions were as recommended by the manufacturer.

Results

Effects of Disruption of *HIF-1α* or *HIF-2α* on Xenograft Response to Sunitinib Treatment

To determine the effects of specifically inhibiting HIF-1 on tumor response to sunitinib, we generated a series of human colon cancer cell lines that do not express HIF-1α: HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}}$, HCT15$^{HIF\text{-}1\alpha\text{-}/\text{-}}$, and LOVO$^{HIF\text{-}1\alpha\text{-}/\text{-}}$. Exons 3 and 4 of the *HIF-1α* gene were targeted for disruption by homologous recombination as described previously

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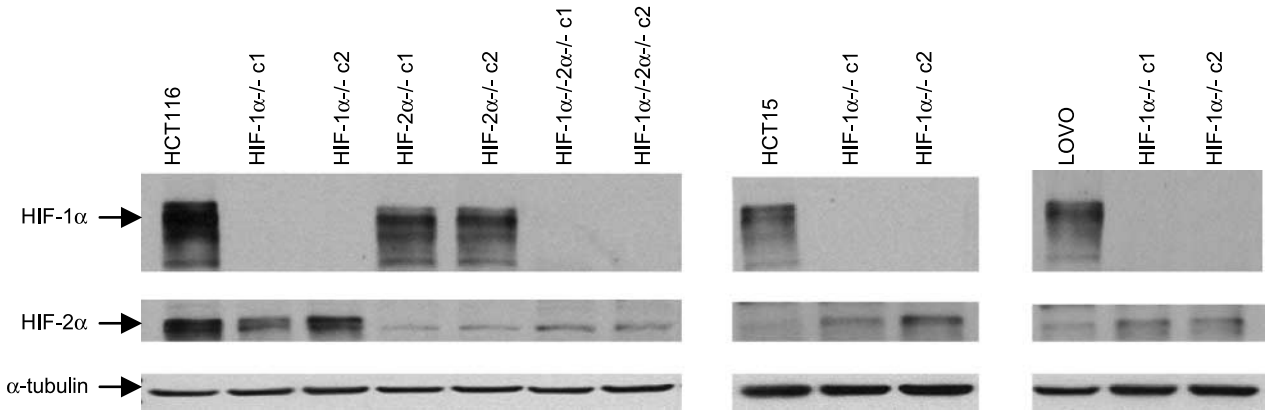

Figure 1. Confirmation of disruption of *HIF-1α* and/or *HIF-2α* by homologous recombination. Parental and disrupted cells were subjected to hypoxia for 24 h and harvested. Western blots using antibodies to HIF-1α and HIF-2α were done, with antibody to α-tubulin as a loading control. *c,* clone.

(43, 44). Complete loss of HIF-1α protein was confirmed by Western blot analysis (Fig. 1). To determine the effects of inhibiting HIF-2 on tumor response to sunitinib, we disrupted the *HIF-2α* gene in HCT116 to generate HCT116$^{HIF\text{-}2\alpha\text{-}/\text{-}}$ cells. Exons 5 and 6 of the *HIF-2α* gene loci were targeted for disruption by homologous recombination as described previously (43, 44). Complete loss of HIF-2α protein was confirmed by Western blot analysis (Fig. 1). The residual lower

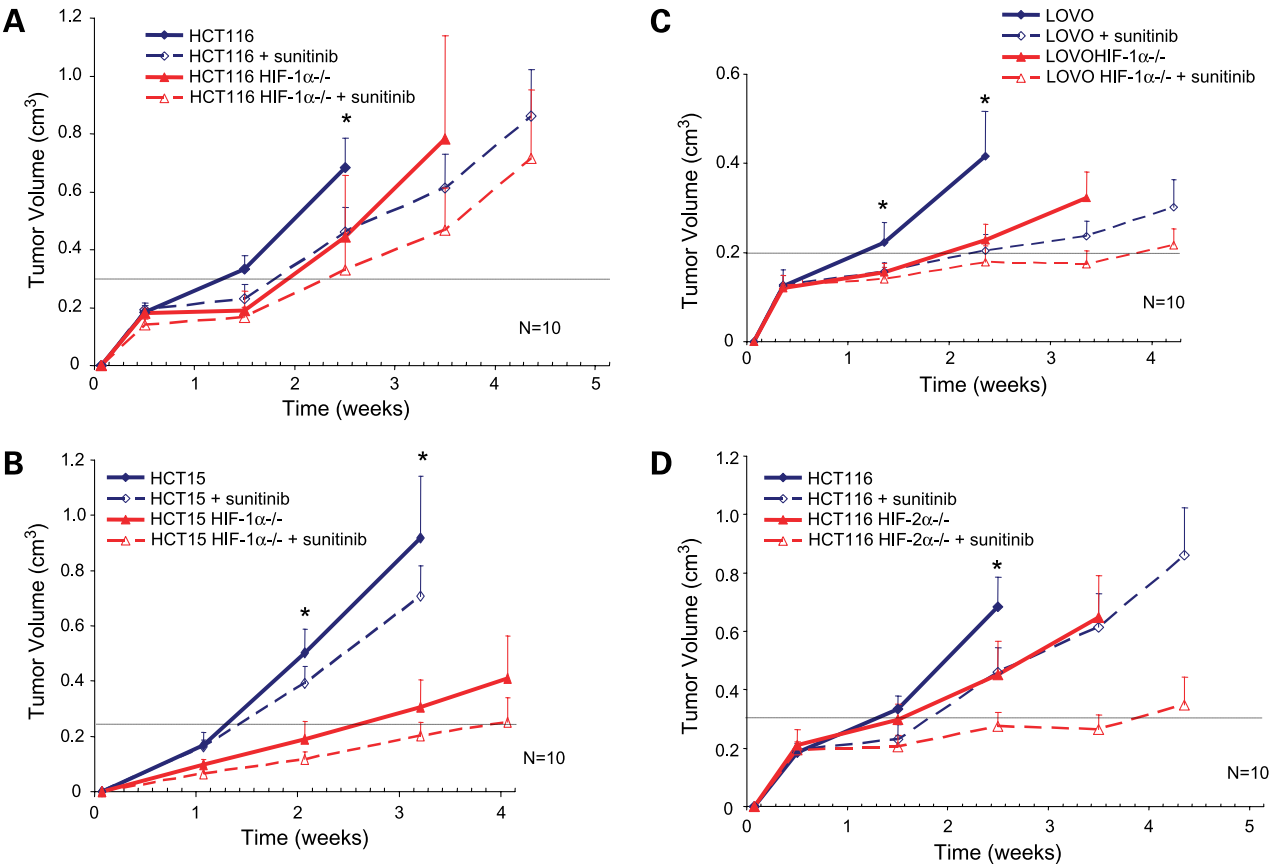

Figure 2. Response to sunitinib treatment in parental, *HIF-1α-/-*, and *HIF-2α-/-* xenografts. Effect of sunitinib in (**A**) HCT116 compared with HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}}$ xenografts, (**B**) HCT15 compared with HCT15$^{HIF\text{-}1\alpha\text{-}/\text{-}}$ xenografts, (**C**) LOVO compared with LOVO$^{HIF\text{-}1\alpha\text{-}/\text{-}}$ xenografts, and (**D**) HCT116 compared with HCT116$^{HIF\text{-}2\alpha\text{-}/\text{-}}$ xenografts. Cells were implanted into the flanks of athymic nude mice and xenograft size was measured and calculated weekly. For sunitinib treatment, tumors were allowed to grow for 2 to 4 d, and mice were treated daily by oral gavage with sunitinib 40 mg/kg/d for 21 d. Control animals were treated with PBS ($n = 10$). *, $P < 0.05$ by Student's t test comparing parental with treatment or knockout xenografts. Multiple knockout clones were tested and showed similar results; for clarity, only one representative clone is presented. Supplementary Fig. S1[5] provides results with two clones.

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bands in the HCT116$^{HIF\text{-}2\alpha\text{-}/\text{-}}$ and HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}HIF\text{-}2\alpha\text{-}/\text{-}}$ cell lysates likely represent nonspecific cross-reactivity of the HIF-2α polyclonal antisera to HIF-1β. Specific loss of HIF-2α function is confirmed by the down-regulation of expression of known HIF-2-regulated genes.[4] Parental and knockout clones were implanted into the flanks of athymic nude mice. After 3 to 5 days, mice were treated with sunitinib by oral gavage, and ensuing xenograft growth was measured.

Disruption of *HIF-1α* led to growth delays in the three human colon cancer xenografts tested. HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}}$ xenografts had a ~4.5-day growth delay compared with HCT116 xenografts, HCT15$^{HIF\text{-}1\alpha\text{-}/\text{-}}$ xenografts had a ~10-day growth delay compared with HCT15 xenografts, and LOVO$^{HIF\text{-}1\alpha\text{-}/\text{-}}$ xenografts had a ~5-day growth delay compared with LOVO xenografts (Fig. 2A-C; Table 1). Disruption of *HIF-2α-/-* led to ~1.5-day growth delay in HCT116$^{HIF\text{-}2\alpha\text{-}/\text{-}}$ xenografts compared with HCT116 xenografts (Fig. 2D; Table 1).

Sunitinib treatment alone led to growth delays in the three parental xenografts, with additional delay in their respective *HIF-1α-/-* or *HIF-2α-/-* xenografts. Sunitinib therapy delayed growth in HCT116 xenografts by ~4 days (Fig. 2A; Table 1). In HIF-1α or HIF-2α disrupted xenografts, sunitinib delayed growth to ~7.5 and ~18 days, respectively (Fig. 2A and D; Table 1). This additive inhibition was also observed in the HCT15 and LOVO series of cells (Fig. 2B and C; Table 1).

We speculated that the additive inhibitory effect on xenografts growth was due to improved inhibition of tumor angiogenesis and/or tumor epithelial cells growth. To evaluate tumor blood vessels, tumor angiogenesis and microvessel perfusion were assessed. In HCT116 xenografts, sunitinib treatment significantly decreased both tumor angiogenesis and microvessel perfusion, respectively (Fig. 3). When compared with sunitinib treatment alone, the combination of sunitinib treatment and disruption of *HIF-1α* did not lead to further decreases in tumor angiogenesis and microvessel perfusion (Fig. 3). These findings were replicated in the HCT15 and LOVO series of cells (Fig. 3). Similarly, when sunitinib treatment was combined with disruption of *HIF-2α*, there was only a slight tumor blood vessel response when compared with sunitinib treatment alone (Fig. 3). Thus, although the combination of sunitinib treatment plus disruption of *HIF-1α* or *HIF-2α* additively inhibits tumor xenograft growth, the combination does not additively inhibit tumor angiogenesis and microvessel perfusion (Figs. 2 and 3). This suggests that the underlying mechanism of additive inhibition might also involve alterations in tumor epithelial cell growth and is examined below in Fig. 4.

Effects of Disruption of Both *HIF-α*s on Xenograft Response to Sunitinib Treatment

HIF-1α and HIF-2α have been shown to share partial structural and functional similarities and bind to HIF-1β to form the HIF-1 and HIF-2 heterodimers, respectively (41, 42, 45–48). HIF-1 and HIF-2 bind to the same hypoxia response element promoter motif and transactivate both unique and overlapping sets of genes, important for tumor angiogenesis and tumor cell growth and survival (42, 49, 50). For these reasons, we hypothesized that combined inhibition of both HIF-1 and HIF-2 would further improve response to sunitinib.

To determine the effects of inhibiting both HIF-1 and HIF-2 on tumor response to sunitinib, we disrupted both *HIF-α*s genes in HCT116 cells to generate the HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}HIF\text{-}2\alpha\text{-}/\text{-}}$ cells. Complete loss of both HIF-1α and HIF-2α proteins was confirmed by Western blot analysis (Fig. 1). Disruption of both *HIF-α*s led to >30-day xenograft growth delay (Fig. 4A; Table 1). Sunitinib treatment of HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}HIF\text{-}2\alpha\text{-}/\text{-}}$ xenografts further delayed their growth, with sustained complete remission in 50% of mice (Fig. 4A; Table 1). We next examined the effects on tumor angiogenesis and microvessel perfusion. Disruption of both *HIF-α*s led to a significant decrease in both tumor angiogenesis and microvessel perfusion (Fig. 3). Combining sunitinib treatment with disruption of *HIF-α*s led to an almost complete inhibition of tumor angiogenesis and microvessel perfusion (Fig. 3).

Because both HIF-1 and HIF-2 are well known to induce VEGF production, we sought to determine if more efficient inhibition of VEGF was responsible for the enhanced antiangiogenic response to sunitinib in the *HIF-α*s disrupted xenografts. Indeed, *VEGF* expression was partially decreased in HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}}$ and HCT116$^{HIF\text{-}2\alpha\text{-}/\text{-}}$ xenografts and further decreased in HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}HIF\text{-}2\alpha\text{-}/\text{-}}$ xenografts when compared with HCT116 xenografts (Fig. 4C).

Table 1. Xenograft growth delay

Xenograft	Growth delay (d)
HCT116 to 0.3 cm^3	0.0
HCT116 + sunitinib	4.0
HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}}$	4.5
HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}}$ + sunitinib	7.5
HCT116$^{HIF\text{-}2\alpha\text{-}/\text{-}}$	1.5
HCT116$^{HIF\text{-}2\alpha\text{-}/\text{-}}$ + sunitinib	18.0
HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}HIF\text{-}2\alpha\text{-}/\text{-}}$	>30
HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}HIF\text{-}2\alpha\text{-}/\text{-}}$ + sunitinib	>30*
HCT116$^{VEGF\text{-}/\text{-}}$	>30
HCT116$^{VEGF\text{-}/\text{-}}$ + sunitinib	>30
HCT15 to 0.25 cm^3	0.0
HCT15 + sunitinib	1.0
HCT15$^{HIF\text{-}1\alpha\text{-}/\text{-}}$	10.0
HCT15$^{HIF\text{-}1\alpha\text{-}/\text{-}}$ + sunitinib	19.0
LOVO to 0.2 cm^3	0.0
LOVO + sunitinib	7.0
LOVO$^{HIF\text{-}1\alpha\text{-}/\text{-}}$	5.0
LOVO$^{HIF\text{-}1\alpha\text{-}/\text{-}}$ + sunitinib	18.0

NOTE: Xenograft growth delay was calculated as days for sunitinib-treated or knockout xenograft to reach designated size compared with parental xenografts. The designated sizes are listed in the table and shown graphically in Figs. 2 and 4.

*Complete remission in 50% of mice.

[4]Article in preparation.

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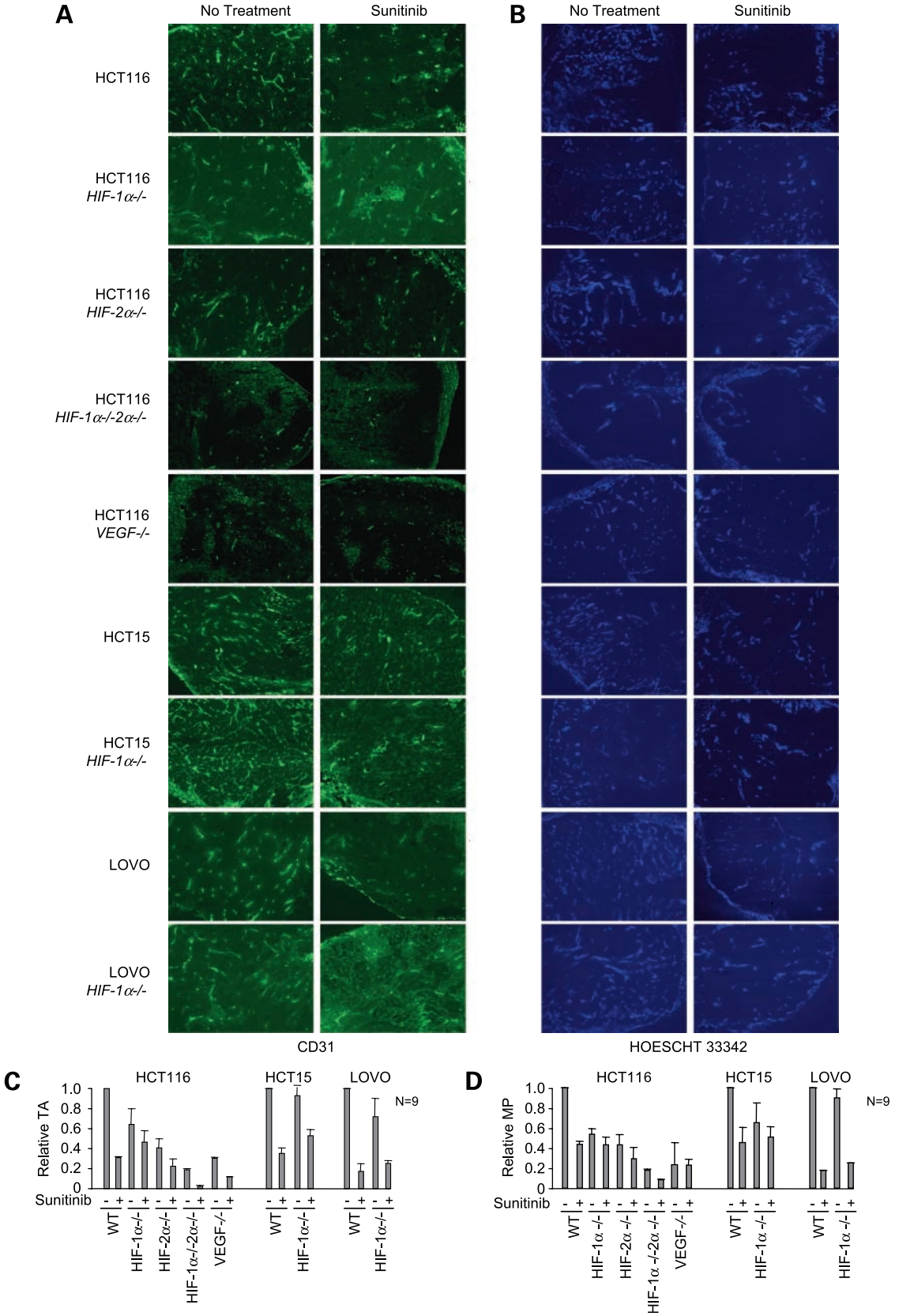

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To more directly test whether superior inhibition of VEGF enhanced tumor response to sunitinib, we disrupted the *VEGF* gene in HCT116 cells by homologous recombination. *VEGF* disruption delayed tumor growth by >30 days in HCT116$^{VEGF-/-}$ compared with their parental counterparts (Fig. 4B). However, addition of sunitinib did not further delay *VEGF-/-* xenograft growth (Fig. 4B). Tumor vessel analyses show that *VEGF* disruption significantly decreased both tumor angiogenesis and microvessel perfusion (Fig. 3). Notably, the decrease in tumor angiogenesis and microvessel perfusion in *VEGF-/-* xenografts was less pronounced than that observed in *HIF-1α-/-HIF-2α-/-* xenografts (Fig. 3). Sunitinib treatment of HCT116$^{VEGF-/-}$ xenografts also resulted in a lesser decrease in tumor angiogenesis and microvessel perfusion compared with sunitinib treatment of HCT116$^{HIF-1\alpha-/-HIF-2\alpha-/-}$ xenografts (Fig. 3). These data suggest that the enhanced antiangiogenic effects from combining *HIF-α*s disruption and sunitinib treatment are not due to the inhibition of VEGF alone.

We next sought to determine whether additional angiogenic factors are regulated by HIF-1 and HIF-2. To systematically identify putative target genes, HCT116 and HCT116$^{HIF-1\alpha-/-HIF-2\alpha-/-}$ xenografts were subjected to global gene expression analyses using an Affymetrix U133A GeneChip. Genes with at least a 4-fold change in expression were identified. As target genes are regulated by the binding of HIF-1 and/or HIF-2 to hypoxia response elements, the genes were further filtered by the existence of the hypoxia response element sequence [(A/G)CGTG] in their promoters.

Disruption of *HIF-1α* and *HIF-2α* led to decreased expression of the gene encoding the proangiogenic factor *angiopoietin-like protein 4* (*ANGPTL4*) and increased expression of the gene encoding the antiangiogenic factor thrombospondin 1 (*TSP1*). To validate these microarray results, *ANGPTL4* and *TSP1* mRNA expression was measured in tumor xenografts by real-time reverse transcription-PCR. Compared with parental HCT116 xenografts, *ANGPTL4* expression was decreased by ~8-fold in HCT116$^{HIF-1\alpha-/-HIF-2\alpha-/-}$ xenografts (Fig. 4C). Conversely, compared with parental HCT116 xenografts, *TSP1* expression was increased by ~8-fold in HCT116$^{HIF-1\alpha-/-HIF-2\alpha-/-}$ xenografts (Fig. 4C). These data show that HIF-1 and HIF-2 inhibition not only down-regulate VEGF but also down-regulate another proangiogenic factor, ANGPTL4, and also up-regulate the antiangiogenic factor, TSP1. As such, antiangiogenic response is superior when sunitinib is combined with HIF-1 and HIF-2 inhibition compared with VEGF inhibition, because the combination disarms multiple angiogenic pathways.

Effects of Disruption of *HIF-1α*, *HIF-2α*, and both *HIF-α*s and Sunitinib Treatment on Tumor Epithelial Cells

Disruption of HIF-1α or HIF-2α enhanced tumor response to sunitinib but did not significantly alter tumor blood vessel response (Figs. 2 and 3). This suggested that disruption of HIF-1α or HIF-2α might directly affect tumor epithelial cell growth.

To determine the effects on epithelial cell growth, cell proliferation and clonogenic survival assay were done. In HCT116 cells, disruption of either *HIF-1α* or *HIF-2α* led to decreased cell growth, and disruption of both *HIF-α*s further decreased growth (Fig. 4D and E). These findings in *HIF-1α-/-* cells were replicated in HCT15 and LOVO cell lines (Supplementary Fig. S2).[5] In the presence of sunitinib at clinically achievable dose range (0.1 μmol/L), no further inhibition of cell growth was observed (Fig. 4D and E). Sunitinib treatment also did not affect cell cycle parameters, rate of DNA synthesis, or cell death (data not shown).

These data illustrated that disruption of *HIF-1α* and *HIF-2α* in colon cancer cells had direct antitumor effect on cell growth, whereas sunitinib treatment did not. As such, targeting HIF-1 and HIF-2 would complement the antiangiogenic effects of sunitinib by directly inhibiting tumor epithelial cells.

Discussion

Altogether, our data show that the antitumor effect of sunitinib treatment on colon cancer xenografts is predominantly mediated by inhibition of tumor angiogenesis. In combination with sunitinib, disruption of both *HIF-1α* and *HIF-2α* additively inhibited tumorigenesis through two mechanisms. The first mechanism is by modulation of additional angiogenic pathways that complement those affected by sunitinib. The second mechanism is through the direct effects of *HIF-1α* and *HIF-2α* on tumor epithelial cell growth. We have shown previously that HIF-1α promotes cell proliferation in the nonhypoxic compartment of tumor xenografts (43). Based on our *in vitro* data, HIF-2α also promotes cell growth under nonhypoxic conditions. As the nonhypoxic compartment is where tumor cells continue to proliferate because of ongoing delivery of oxygen and nutrients, this compartment might allow for tumor escape despite antiangiogenic therapy. Therefore, targeting tumor epithelial cells

[5] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Figure 3. Tumor vessel response to sunitinib treatment in parental, *HIF-1α-/-*, *HIF-2α-/-*, and *HIF-1α-/-HIF-2α-/-* xenografts. **A,** tumor angiogenesis (*TA*). **B,** tumor microvessel perfusion (*MP*). Parental and knockout xenografts were treated with placebo or sunitinib 40 mg/kg/d for 21 d, harvested, and fixed. For tumor angiogenesis, microvessel density was determined by staining sections with a monoclonal antibody against the endothelial cell-specific marker CD31. For tumor microvessel perfusion studies, mice were injected i.v. with Hoechst 33342 at 2 min before sacrifice. Tumors were fixed, sectioned, and examined under fluorescence microscopy. Representative photomicrographs were obtained. Original magnification, ×40. Quantification of (**C**) tumor angiogenesis and (**D**) tumor microvessel perfusion. Images from three representative sections from three different tumors were quantified using Image-Pro Plus software (n = 9). Tumor angiogenesis and microvessel perfusion are depicted as relative to wild-type untreated xenografts.

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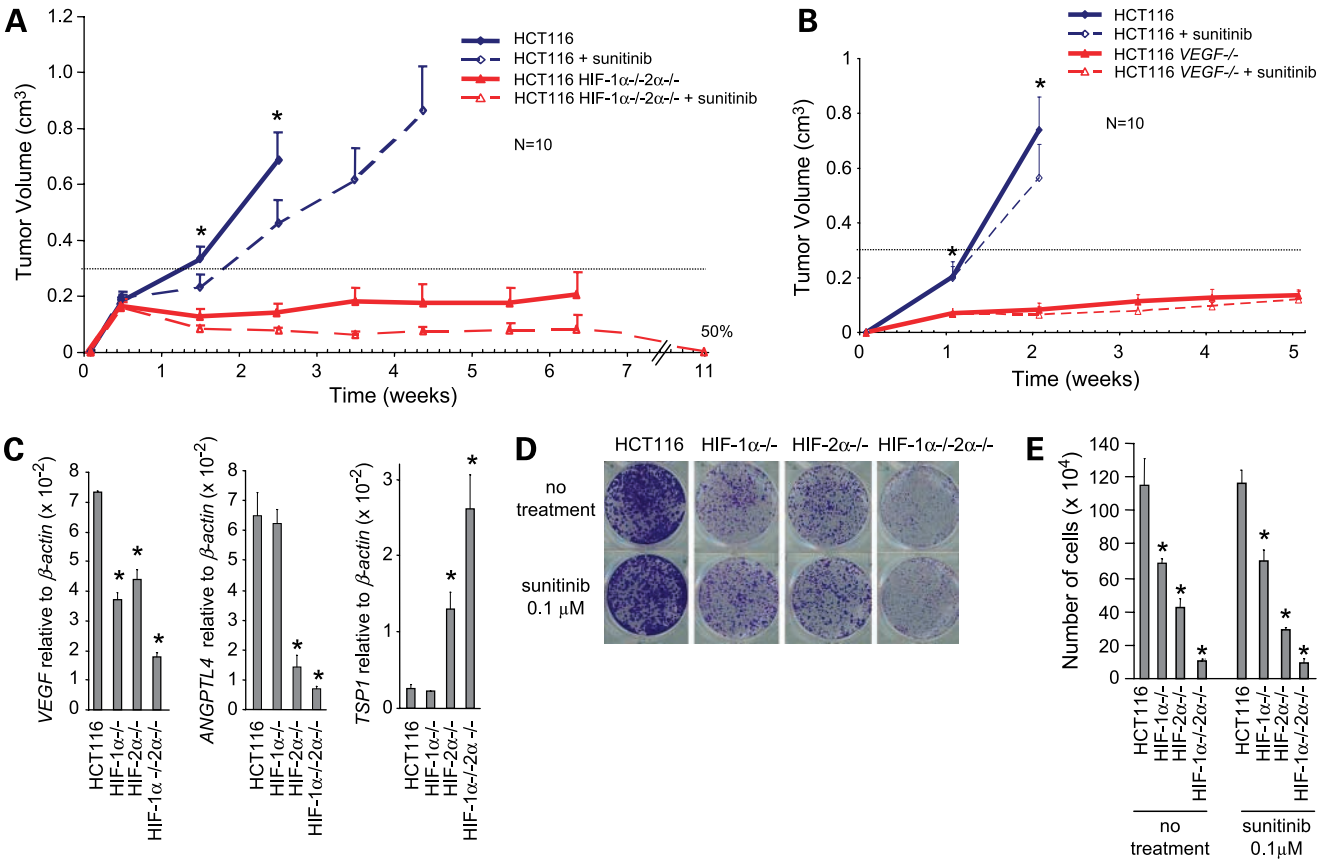

Figure 4. Response to sunitinib treatment in parental, *HIF-1α-/-HIF-2α-/-*, and *VEGF-/-* xenografts. Effect of sunitinib in (**A**) HCT116 compared with HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}HIF\text{-}2\alpha\text{-}/\text{-}}$ xenografts and (**B**) HCT116 compared with HCT116$^{VEGF\text{-}/\text{-}}$ xenografts. Cells were implanted into the flanks of athymic nude mice and xenograft size was measured and calculated weekly. For sunitinib treatment, tumors were allowed to grow for 2 to 4 d, and mice were treated daily by oral gavage with sunitinib 40 mg/kg/d for 21 d. Control animals were treated with PBS ($n = 10$). *, $P < 0.05$ by Student's t test comparing parental with treatment or knockout xenografts. **C,** induction of angiogenic factors in xenografts. RNA was harvested from xenografts and subjected to real-time reverse transcription-PCR with primers for *VEGF, ANGPTL4, TSP1*, and *β-actin*. Gene expressions relative to *β-actin* are graphed ($n = 9$). *, $P < 0.05$ by Student's t test comparing treatment or knockout with parental xenografts. Multiple knockout clones were tested and showed similar results; for clarity, only one representative clone is presented. **D,** colony formation in response to sunitinib treatment in parental, *HIF-1α-/-*, *HIF-2α-/-*, and *HIF-1α-/-HIF-2α-/-* cell lines. Cancer cells were plated in the presence or absence of sunitinib and allowed to grow undisturbed for 10 d. Colony formation was determined by staining with crystal violet. Representative photomicrographs were obtained. Multiple knockout clones were tested and showed similar results; for clarity, only one representative clone is presented. **E,** cell proliferation in response to sunitinib treatment in parental, *HIF-1α-/-*, *HIF-2α-/-*, and *HIF-1α-/-HIF-2α-/-* cell lines. Cancer cells were plated in the presence or absence of sunitinib, incubated under normoxic conditions, and then counted daily for 7 d after trypan blue exclusion. Data are shown for counts at day 7 ($n = 3$). *, $P < 0.01$ by Student's t test comparing parental with *HIF-1α-/-*, *HIF-2α-/-*, and *HIF-1α-/-HIF-2α-/-* cells.

directly through inhibition of HIF-1α and/or HIF-2α would complement the antiangiogenic effects of sunitinib.

Our preclinical findings suggest an approach in which sunitinib may be combined with inhibitors of HIF-1 and/or HIF-2 for cancer treatment. Combining sunitinib with HIF-1 and/or HIF-2 inhibition led to additive inhibitory effects in xenografts derived from multiple cell lines. These preclinical findings suggest that this combination in patients would also lead to additive clinical benefit, with improved clinical endpoints including progression-free survival, time to progression, and likely overall survival.

To date, high-throughput small-compound screens and mechanistic studies have identified several classes of anticancer agents that disrupt HIF-1 function, including inhibition of its transcriptional activity and HIF-1α protein synthesis or stability (39, 51–56). Additional screening is ongoing to identify inhibitors of HIF-1α and HIF-1β interactions (57). Based on partial structural and functional similarities between HIF-1 and HIF-2, it is possible that many of the already identified HIF-1 inhibitors would inhibit HIF-2. Our data suggest that it may be advantageous to systematically identify compounds that are effective at inhibiting both HIF-1 and HIF-2 for cancer treatment.

The improved antiangiogenic effect of *HIF-α*s disruption plus sunitinib treatment is not mediated by an enhanced inhibition of the VEGF/VEGFR axis but likely by the inhibition of another proangiogenic factor, ANGPTL4, and induction of the antiangiogenic factor, TSP1. Our data are consistent with previous preclinical studies showing compensatory induction of other proangiogenic factors in

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addition to VEGF on initiation of antiangiogenic therapy (17, 18). These data also suggest that combining sunitinib and agents primarily targeting the VEGF pathway might not improve clinical response or benefit. A potential advantage of combining sunitinib with agents targeting HIF-1 and HIF-2 is their effects on complementary angiogenic factors.

In conclusion, our study shows that effective antitumor treatment may be achieved by maximal inhibition of both tumor blood vessel and tumor epithelial cell compartments. However, important questions remain, and further investigations are ongoing in our laboratory. First, the mechanism by which HIF-1 and HIF-2 coordinate and induce tumor angiogenesis through the regulation of proangiogenic factor(s) and antiangiogenic factor(s) remains to be determined. Second, it is not entirely known how HIF-1 and HIF-2 interact and regulate cancer cell growth and survival. It is likely that the mechanisms by which HIF-1 and HIF-2 regulate cell growth and survival are unique and overlapping, and target genes controlling nutrients metabolism, growth, and survival pathways need to be identified. Further mechanistic studies in these areas will likely yield additional targets for drug development and novel combinations for cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Targeting both HIF-1 and HIF-2 in human colon cancer cells improves tumor response to sunitinib treatment

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