Acriflavine inhibits HIF-1 dimerization, tumor growth, and vascularization

Kang Ae Lee a,b, Huafeng Zhang a,b, David Z. Qian a,b, Sergio Rey a,b, Jun O. Liu c,d, and Gregg L. Semenza a,b,c,e,1

a Vascular Program, Institute for Cell Engineering, b McKusick–Nathans Institute of Genetic Medicine, Departments of Oncology, c Pharmacology, and d Pediatrics, Medicine, Radiation Oncology, and Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

Contributed by Gregg L. Semenza, August 18, 2009 (sent for review July 17, 2009)

HIF-1 is a heterodimeric transcription factor that mediates adaptive responses to hypoxia and plays critical roles in cancer progression. Using a cell-based screening assay we have identified acriflavine as a drug that binds directly to HIF-1α and HIF-2α and inhibits HIF-1 dimerization and transcriptional activity. Pretreatment of mice bearing prostate cancer xenografts with acriflavine prevented tumor growth and treatment of mice bearing established tumors resulted in growth arrest. Acriflavine treatment inhibited intratumoral expression of angiogenic cytokines, mobilization of angiogenic cells into peripheral blood, and tumor vascularization. These results provide proof of principle that small molecules can inhibit dimerization of HIF-1 and have potent inhibitory effects on tumor growth and vascularization.

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oild tumors frequently contain hypoxic regions because they have high rates of cell proliferation and form aberrant blood vessels (1). Intratumoral hypoxia is associated with increased risk of invasion, metastasis, and patient mortality (2). The adaptation of cancer cells to hypoxia is critical for their survival. Hypoxia-inducible factor 1 (HIF-1) activates transcription of genes encoding proteins that mediate major adaptive responses to hypoxia (2). For example, HIF-1 activates the expression of vascular endothelial growth factor (VEGF), a key regulator of angiogenesis, as well as glucose transporters (e.g., GLUT1) and glycolytic enzymes (e.g., hexokinase [HK] 1 and 2), which are required for high levels of glucose uptake and metabolism (2). The fact that HIF-1 regulates the expression of multiple gene products involved in tumor metabolism and vascularization suggests that a greater anticancer effect may be achieved by inhibition of HIF-1, as compared to a downstream gene product, such as VEGF.

HIF-1 is a heterodimeric protein that is composed of HIF-1α and HIF-1β subunits, which belong to the family of basic helix-loop-helix (bHLH) transcription factors that contain a PER-ARNT-SIM (PAS) domain (3). In contrast to the constitutively expressed HIF-1β subunit, high levels of HIF-1α are induced in response to hypoxia. HIF-1α is constantly synthesized and, in well-oxygenated cells, is hydroxylated on proline residue 402 and/or 564, which is required for binding of the von Hippel-Lindau protein, the recognition subunit of an E3 ubiquitin ligase that targets HIF-1α for protemporal degradation (4). Asparagine 803 is also hydroxylated, which inhibits recruitment of the coactivator proteins p300 and CREB binding protein (CBP) to the transcriptional activation domain of HIF-1α. The prolyl and asparaginyl hydroxylases require O2 for their catalytic activity. Under hypoxic conditions, hydroxyla- 

tion decreases, HIF-1α accumulates and dimerizes with HIF-1β to form a functional transcription factor capable of DNA binding at hypoxia response elements (HREs) and transcriptional activation. HIF-2α is another bHLH-PAS protein that is O2-regulated, dimerizes with HIF-1α, and binds to HREs (4).

Increased HIF-1α or HIF-2α levels are found in human lung, colon, breast, and prostate carcinomas, and are associated with disease progression and increased patient mortality (2). A large body of experimental data indicates that disruption of HIF-1 signaling inhibits tumor growth in mouse models (2). A growing number of drugs have been identified that (i) inhibit HIF-1 activity through a reduction in HIF-1 mRNA or protein levels, HIF-1 DNA binding, or transactivation of target genes; and (ii) have anticancer activity in preclinical studies (5). However, no drugs have been identified that bind directly to HIF-1.

Dimerization of HIF-1β with HIF-1α or HIF-2α, which is required for HIF-1 DNA binding and transcriptional activity, is mediated by bHLH and PAS domains located in the amino-terminal half of each subunit (3, 6). A small molecule that targets HIF-1 dimerization might function as a selective HIF-1 inhibitor, but current pharmacological dogma holds that small molecules are unlikely to disrupt large dimerization interfaces, such as the combined bHLH and PAS domains, which span over 200 amino acids (aa). In this study, we identified an inhibitor of HIF-1 dimerization, which decreased HIF-1 transcriptional activity and showed anticancer efficacy in vivo that was due at least in part to its antiangiogenic effects.

Results

Screening for Inhibitors of HIF-1 Dimerization. We developed a cell-based dimerization assay based on complementation of split Renilla luciferase (Rluc) (7). In this case, dimerization of the HIF-1α and HIF-1β bHLH-PAS domains provided the mechanism for complementation (Fig. 1A). Interaction of HIF-1α(12–396) and HIF-1β11–510 causes the N- and C-terminal halves of Rluc to be closely approximated, thereby reconstituting Rluc activity. Vectors encoding NRLuc-HIF-1α(12–396) and HIF-1β11–510-CRLuc fusion proteins were cotransfected with control reporter pGL2-promoter, in which firefly luciferase (Fluc) coding sequences are downstream of an SV40 promoter. Rluc activity was detected in cells expressing NRLuc-HIF-1α(12–396) and HIF-1β11–510-CRLuc, but no Rluc activity was observed when either NRLuc-HIF-1α(12–396) or HIF-1β11–510 CRLuc was expressed alone (Fig. 1B).

To validate the specificity of the split Rluc assay, we coexpressed NRLuc-HIF-1β11–510 and found that it competes with HIF-1β11–510-CRLuc for binding to NRLuc-HIF-1α(12–396), and thereby blocks reconstitution of Rluc activity (SI Appendix, Fig. S1A). We also coexpressed a double-mutant form of HIF-1α (HIF-1αDM) that contains Pro-to-Ala substitutions at residues 402 and 564, thereby preventing hydroxylation and rendering HIF-1α stable under nonhypoxic conditions. HIF-1αDM competed with NRLuc-HIF-1α(12–396) for dimerization with HIF-1β11–510-CRLuc (SI Appendix, Fig. S1B). The finding that increasing concentrations of NRLuc-HIF-1α(12–396) or HIF-1αDM led to decreasing Rluc activity confirmed that Rluc activity was dependent on NRLuc-HIF-1α(12–396)/HIF-1β11–510-CRLuc interaction and indicated that the assay was suitable for identifying inhibitors of HIF-1 dimerization.


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1To whom correspondence should be addressed. E-mail: gsemenza@jhmi.edu.

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IC50 of approximately 1

R

HIF-1

1

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for 24 h. IP with anti-HIF-1

To confirm that ACF inhibits dimerization of HIF-1

identified 336 drugs that inhibited hypoxia-induced transcription

approved by the FDA or have entered phase II clinical trials and

Fig. 1. Split R luc system for identifying inhibitors of HIF-1 dimerization. (A) N-terminal and C-terminal portions of R luc were attached to HIF-1α12–396 and HIF-1α11–510, respectively. (B) The ratio of Renilla/luciferase activity (R luc/R luc) was determined using cells co-transfected with pGL2-promoter, which encodes R luc, and N/R luc-HIF-1α12–396 (NR-1α) and/or HIF-1α11–510-CR/R luc (1α-CR). Each value was normalized to the results for empty vector (EV). Data represent mean ± SEM (n = 6). **, P < 0.01 (Student’s t test) vs. EV. C, HEK293 cells were cotransfected with pGL2-promoter, NR-1α, and 1α-CR vectors and treated with acriflavin (ACF) for 24 h. The R luc/R luc ratio in cell lysates was determined and normalized to the results for NR-1α. Mean ± SEM are shown (n = 6). *, P < 0.05; **, P < 0.01 vs. 0 μM ACF (Student’s t test).

Fig. 1A

Dimerization

I

N-Ruc

C-Ruc

Active Ruc

R luc/Fluc

HeLa

ACF (μM)

1

0.2

0

0.5

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R luc/Fluc

ACF (μM)

30

20

10

0

NR-1α + 1α-CR

0

R luc/Fluc

ACF (μM)

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We previously screened a collection of 3,120 drugs that are approved by the FDA or have entered phase II clinical trials and identified 336 drugs that inhibited hypoxia-induced transcription of HIF-1-dependent reporter plasmid p2.1 by >50% at a concentration of 10 μM (8). The top 200 hits were subjected to secondary screening using the split R luc assay. The most potent inhibitor of HIF-1 dimerization was acriflavin (ACF), which inhibited R luc activity by 94% at a concentration of 5 μM (Fig. 1C). ACF inhibited R luc in a dose-dependent manner with an IC50 of approximately 1 μM.

Acriflavin Inhibits the Dimerization of HIF-1α (or HIF-2α) with HIF-1β.

To confirm that ACF inhibits dimerization of HIF-1α and HIF-1β, we performed coimmunoprecipitation (co-IP) assays. HEK293 cells were treated with ACF or vehicle and exposed to 20 or 1% O2 for 24 h. IP with anti-HIF-1α antibodies (Ab) showed that ACF decreased interaction between endogenous HIF-1α and HIF-1β in hypoxic cells (Fig. 2A). We also incubated a purified GST-HIF-1β11–510 fusion protein with lysates from cells transfected with Flag-HIF-1αDM (Fig. 2B). Flag-HIF-1αDM was pulled down with GST-HIF-1β11–510, but not with GST alone, and the interaction was decreased when cells were treated with ACF. ACF also decreased the interaction between HIF-2α and endogenous HIF-1β (SI Appendix, Fig. S2A) or purified GST-HIF-1β11–510 (SI Appendix, Fig. S2B).

In vitro binding assay was also performed using purified GST-HIF-1β11–510 and His-HIF-1α12–395 (or His-HIF-2α3–350) proteins. His-HIF-1α12–395 (Fig. 2C) or His-HIF-2α3–351 (SI Appendix, Fig. S2C) was pulled down by GST-HIF-1β11–510, but not by GST alone, and these interactions were inhibited by ACF. The ability of ACF to disrupt HIF-1 (Fig. 2D) or HIF-2 (SI Appendix, Fig. S2D) dimerization was dependent on the concentration of ACF. The observed IC50 was approximately 1 μM and 5 μM led to almost complete inhibition.

Acriflavine Binds Directly to HIF-1α and HIF-2α. To determine whether ACF binds to HIF-1β, purified GST-HIF-1β11–510 was preincubated with ACF and the complex was captured by glutathione beads, washed 1–3 times to eliminate unbound ACF, and then incubated with purified His-HIF-1α3–350. Preincubation of GST-HIF-1β11–510 with ACF did not inhibit dimerization of GST-HIF-1β11–510 and His-HIF-1α12–395 when the preincubated complex was washed extensively (Fig. 3C). Moreover, His-HIF-1α12–395 levels captured by GST-HIF-1β11–510 did not decrease with increasing concentration of ACF during preincubation (Fig. 3B). These results indicate that HIF-1β is not a target of ACF. Next, purified His-HIF-1α12–395 was preincubated with ACF, captured by Ni-NTA-agarose beads, washed, and incubated with GST-HIF-1β11–510. Preincubation of His-HIF-1α12–395 with ACF decreased binding of GST-HIF-1β11–510 even after extensive washing (Fig. 3C). This effect was dependent on the ACF concentration during preincubation (Fig. 3D). These results indicate that ACF binds to HIF-1α and disrupts its interaction with HIF-1β.

To confirm these conclusions, we determined the direct binding affinity of ACF to HIF-1α or HIF-2α by following the innate fluorescence of ACF at λex = 463 nm and λem = 490 nm (SI Appendix, Fig. S3). Purified GST-HIF-1α3–350 or GST-HIF-2α3–351 was incubated with 0–250 μM ACF and captured by glutathione agarose beads. The protein-drug complex was washed 3 times and analyzed for fluorescence intensity (FI; Fig. 3E). The FI of GST-HIF-1α3–350 or GST-HIF-2α3–351 increased in a dose-dependent manner as ACF concentration increased, whereas neither GST nor GST-HIF-1α3–351 showed increased FI even at high ACF concentrations. A significant difference between HIF-1α subunits and control proteins was first observed at 1 μM ACF (Fig. 3E Insert) and FI was maximal at 100 μM.

Acriflavine Specifically Binds to PAS-B Subdomain of HIF-1α and HIF-2α. We tested purified proteins that contained different domains of HIF-1α for ACF binding (Fig. 4A and SI Appendix, Fig. S4 A and B). ACF increased the FI of all GST fusion proteins that contained the PAS-B domain, whereas no difference in FI was observed after ACF vs. vehicle treatment of all fusion proteins that lacked PAS-B. Maximal FI was captured by GST-HIF-1α325–350, which contained only PAS-B, whereas FI was not captured by GST-HIF-1α290–355, which contained only PAS-A. Similar results were also observed for HIF-2α (Fig. 4B and SI Appendix, Fig. S4 C and D). For both HIF-1α and HIF-2α, a significant difference in captured FI between fusion proteins that included vs. excluded PAS-B was first observed at a concentration of 1 μM ACF (SI Appendix, Fig. S4 B and D Insert). These results indicate that ACF interacts specifically with the PAS-B subdomain of HIF-1α or HIF-2α.

The aryl hydrocarbon receptor (AHR) is a bHLH-PAS transcription factor that also heterodimerizes with HIF-1β. The binding of ACF to GST-fusion proteins containing AHR residues 1–410 (bHLH-PAS) or 281–410 (PAS-B) was not significantly different from GST alone (SI Appendix, Fig. S5), indicating that ACF does not bind to AHR. To test whether ACF blocks heterodimerization...
by inducing HIF-1α homodimerization, purified GST-HIF-1α3-350 or GST-HIF-1β11-510 was incubated with lysates obtained from HEK293 cells exposed to 1% O2 in the presence of ACF or vehicle. HIF-1α was captured by GST-HIF-1β11-510 and ACF decreased the interaction between HIF-1α and GST-HIF-1β11-510 whereas no HIF-1α was captured by GST-HIF-1α3-350 in the presence of ACF (SI Appendix, Fig. S6A). His-HIF-1α3-350 also failed to capture GST-HIF-1α3-350 in the presence of ACF (SI Appendix, Fig. S6 B and C). Thus, ACF does not induce homodimerization of HIF-1α.

Acriflavine Inhibits HIF-1 DNA Binding and Transcriptional Activity. To investigate the functional consequences of disrupting HIF-1 dimerization, we first determined the effect of ACF on the binding of endogenous HIF-1 to DNA in living cells. Chromatin-bound HIF-1α was immunoprecipitated from HEK293 cells after exposure to 20% or 1% O2 in the presence of 0–10 μM ACF. HIF-1 binding sites from the VEGF and PDK1 genes were specifically amplified by PCR from chromatin that was immunoprecipitated from cells exposed to 1% O2 but not from chromatin that was immunoprecipitated from cells exposed to 20% O2, demonstrating hypoxia-induced binding of HIF-1 in the absence of drug (Fig. S4). Treatment of hypoxic cells with ACF inhibited binding of HIF-1α to DNA in a dose-dependent manner. ACF also blocked binding of HIF-2α to the VEGF and PDK1 genes in hypoxic cells (SI Appendix, Fig. S7). We next determined the effect of ACF on HIF-1 transcriptional activity. HEK293 cells were cotransfected with HIF-1α-dependent Fluc reporter p2.1 and pSV-Renilla, and exposed to 20% or 1% O2. ACF significantly inhibited the hypoxic induction of Fluc activity in a dose-dependent manner with an IC50 of approximately 1 μM and complete inhibition at 5 μM (Fig. S5B). Hypoxic induction of VEGF and GLUT1 mRNA was also blocked by ACF (Fig. S5C). ACF did not affect HIF-1α protein accumulation (Fig. S5D).

Acriflavine Does Not Affect HIF-1α/Hsp90 Interaction or MYC-mediated Transcription. HIF-1α interacts with the chaperone Hsp90 and disruption of HIF-1α/Hsp90 association leads to proteasomal degradation of HIF-1α (9). Similar levels of Hsp90 were communo-purified with HIF-1α from lysates of hypoxic HEK293 cells treated with ACF or vehicle, indicating that ACF does not disrupt the interaction between HIF-1α and Hsp90 (SI Appendix, Fig. S8A). We also tested whether ACF affects MYC-mediated transcription using human P493 cells with tetracycline-repressible MYC activity (10). Expression of fibrillarin (FBL) and apurinic/apyrimidinic exonuclease (APEX) mRNAs, which are products of MYC target genes, was blocked by tetracycline (SI Appendix, Fig. S8B). ACF did not affect FBL or APEX mRNA levels, whereas ACF specifically decreased hypoxia-induced GLUT1 mRNA expression.

Fig. 2. Inhibition of HIF-1 dimerization by ACF. (A) Effect of ACF on HIF-1α/HIF-1β interaction was determined using communoprecipitation (co-IP). HEK293 cells were treated with ACF or vehicle and exposed to 20% or 1% O2 for 24 h. IP of whole cell lysates (WCL) was performed with anti-HIF-1α antibodies (Ab). WCL and IP products were assayed by immunoblot (IB) to detect HIF-1β, HIF-1α, and β-actin. (B) GST-HIF-1β11-510 was incubated with WCL from HEK293 cells transfected with vector encoding Flag-HIF-1α350 in the presence or absence of ACF. Proteins were pulled down with glutathione-agarose beads and subjected to IB assays using anti-FLAG and anti-GST Ab. An aliquot of WCL was analyzed directly by IB assay (Input). (C) Purified His-HIF-1α3-350 was incubated with purified GST-HIF-1β11-510 or GST in the presence of ACF or vehicle overnight at 4°C. Binding was determined by GST pull down and IB using anti-His and anti-GST Ab. An aliquot of His-fusion protein was analyzed directly by IB assay (Input). (D) Dose-dependent effects of ACF on HIF-1α dimerization in vitro were determined.

Fig. 3. ACF binds directly to HIF-1α and HIF-2α. (A) Purified GST-HIF-1β11-510 was preincubated with vehicle (−) or ACF (+) and the complex was captured by glutathione beads, subjected to three washes, incubated with purified His-HIF-1α3-350, washed again, and proteins bound to glutathione were analyzed by IB using anti-FLAG and anti-GST Ab. ACF specifically decreased hypoxia-induced GLUT1 mRNA expression.

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Acriflavine Inhibits Tumor Xenograft Growth. We examined whether inhibition of HIF-1 dimerization by ACF affects the growth of human cancer xenografts. ACF did not affect HIF-1α or HIF-2α mRNA or protein levels in PC-3 human prostate cancer cells and Hep3B-c1 cells (SI Appendix, Figs. S9 and S10). ACF also did not affect the levels of HIF-1α, c-Myc, Hsp90, or β-Actin protein in PC-3 or Hep3B-c1 cells (SI Appendix, Fig. S10). These data rule out nonspecific effects of ACF on transcription or translation at concentrations that block HIF-1 activity. In contrast, HRE-dependent transcription (SI Appendix, Fig. S9B) and the expression of VEGF, stromal-derived factor 1 (SDF1), stem cell factor (SCF), and GLUT1 mRNAs, which are all encoded by HIF-1 target genes (SI Appendix, Fig. S9C), were significantly inhibited by treatment of PC-3 cells with ACF. ACF had no effect on PC-3, P493, or Hep3B-c1 cell cycling, proliferation, or survival at concentrations that block HIF-1 activity (SI Appendix, Fig. S11).

To investigate the effect of ACF on tumor growth, vehicle or ACF was administered by daily i.p. injection to severe combined immune deficiency (SCID) mice that received s.c. PC-3 cell xenografts. Treatment was initiated 3 days before implantation and continued for 32 days. Tumors were palpable in vehicle-treated mice by day 12 and grew to approximately 500 mm³ by day 32, whereas mice treated with ACF did not show any significant tumor growth even after one month (SI Appendix, Fig. S12). Daily administration of ACF did not cause weight loss (SI Appendix, Fig. S13A), suggesting ACF has no major systemic toxicity.

When treatment was delayed until 14 days after s.c. implantation of PC-3 cells, at which time the tumors had grown to approximately 100 mm³, ACF inhibited tumor growth after 7 days of administration (days 14 through 21), which continued through day 28 (Fig. 6B) without any effect on body weight (SI Appendix, Fig. S13B). Expression of VEGF, SDF1, SCF, GLUT1, HK1, and HK2 mRNAs was decreased in tumors after treatment with ACF (Fig. 6B), whereas HIF-1α protein was highly expressed in tumors from mice treated with vehicle or ACF (SI Appendix, Fig. S14).

Next, Hep3B-c1 hepatoma cells stably transfected with the HIF-1-regulated FLuc reporter gene p2.1 were implanted in SCID mice. Before initiation of treatment (day 25), tumor volume (~100 mm³) was similar across groups (Fig. 6C). Analysis of HIF-1-dependent FLuc activity by whole-body Xenogen imaging revealed bioluminescence at the tumor site that was similar in both groups (Fig. 6D Upper). Mice were then treated with ACF or vehicle by daily i.p. injection and Xenogen imaging was repeated 4 h after treatment on day 28. The bioluminescence in tumors before and after treatment with vehicle was similar. In contrast, FLuc activity was markedly decreased after treatment with ACF for 4 days (Fig. 6D Lower).
VEGFR2 first observed on day 36 (Fig. 6) which preceded the significant inhibition of tumor growth that was similar to those in nontumor bearing mice (Fig. 7). 9 days reduced SDF-1 in the blood of tumor-bearing mice to levels tumor-bearing vs. nontumor-bearing mice and ACF treatment for effect of ACF on CACs, SCID mice bearing PC-3 xenografts of other cell types that home to tumors and stimulate angiogenesis. including endothelial progenitor, mesenchymal stem, myeloid, and genic cells (CACs), a term that designates a heterogeous population marrow and other sites into peripheral blood of circulating angio-

ACF inhibits tumor growth, HIF-1 target gene expression, and HIF-1 activity. (A and B) PC-3 xenografts were grown to approximately 100 mm³ and mice were treated by daily i.p. injection of vehicle or ACF for 14 days. (A) Tumor volume (mean ± SEM; n = 4) is shown. *, P < 0.05; **, P < 0.01 vs. vehicle (two-way ANOVA with Bonferroni correction). (B) Mice were euthanized on day 28 (4 h after last dose) and tumors were collected. mRNA levels relative to 18S rRNA in tumors from vehicle- and ACF-treated mice were calculated as 2(ΔΔCt), where ΔCt = Ct(target) - Ct(ref) and ΔΔCt = ΔCt(vehicle) - ΔCtACF. Mean ± SEM (n = 4) is shown. *, P < 0.05; **, P < 0.01 (two-way ANOVA with Bonferroni correction). (C-D) mice bearing Hep3B-c1 xenografts were treated with vehicle or ACF starting on day 25. (D) HRE-driven FLuc activity was determined by Xenogen imaging before treatment (Upper) and 4 h after treatment on day 28 (Lower).

ACrflavine Inhibits Angiogenic Cell Mobilization and Tumor Vascularization. VEGF, SCF, and SDF1 induce the mobilization from bone marrow and other sites into peripheral blood of circulating angiogenic cells (CACs), a term that designates a heterogeneous population that home to tumors and stimulate angiogenesis. HIF-1 plays a critical role in these processes (11). To determine the effect of ACF on CACs, SCID mice bearing PC-3 xenografts of approximately 50 mm³ were treated with ACF for 9 days (SI Appendix, Fig. S15). Four hours after the last dose, blood was collected for flow cytometric analysis of CACs, as defined by the coexpression of a progenitor marker (CD34, CD117 [c-kit], or Sca1) and either VEGFR2 or CXCR4 (Fig. 7A). The number of VEGFR2+/CD117+, VEGFR2+/CD34+, and CXCR4+/Sca1+ CACs was increased approximately 5-fold in tumor-bearing mice compared with mice without tumors and no significant differences were observed between untreated and vehicle-treated tumor-bearing mice. In contrast, ACF treatment significantly decreased the number of CACs in tumor-bearing mice to levels observed in mice without tumors. ACF inhibited the expression of mRNAs encoding VEGF, SDF1, and SCF (which are the ligands bound by VEGFR2, CXCR4, and CD117, respectively) in PC-3 xenografts (Fig. 6B). SDF-1 protein levels were increased in blood from tumor-bearing vs. nontumor-bearing mice and ACF treatment for 9 days reduced SDF-1 in the blood of tumor-bearing mice to levels similar to those in nontumor bearing mice (Fig. 7B). These effects of ACF were associated with a significant reduction in tumor vascularization (Fig. 7C).

Discussion

There is interest in the discovery of drugs that target HIF-1 because of the critical role that it plays in cancer progression (1, 2, 5). Identifying an inhibitor of HIF-1 dimerization would be particularly desirable as a defined and selective mechanism of action, although conventional wisdom holds that the large interfaces involved in protein dimerization are difficult to disrupt by small molecules. We used a cell-based split-RLuC assay to identify ACF as drug that inhibits HIF-1 dimerization (Figs. 1–3) by binding to the PAS-B subdomain of HIF-1α and HIF-2α (Fig. 4), thereby inhibiting HIF-1 DNA-binding and transcriptional activity (Figs. 5 and 6), leading to inhibition of tumor growth, CAC mobilization, and tumor vascularization (Figs. 6 and 7).

PAS domains of HIF-1α, HIF-2α, HIF-1β, and other bHLH-PAS proteins are organized into PAS-A and PAS-B subdomains, which contribute to dimerization by providing, in addition to the bHLH domain, secondary interaction surfaces that increase the specificity of dimerization. NMR spectroscopy revealed that hydrophobic residues located on the solvent-exposed face of a β-sheet in the HIF-2α PAS-B subdomain mediate heterodimerization with HIF-1β (12). The HIF-2α PAS-B crystal structure was shown to contain an internal cavity, which accommodated a small molecule that partially disrupted the heterodimerization of isolated HIF-2α and HIF-1β PAS subdomains in vitro but was not reported to have any effect on heterodimerization of the full length proteins or on HIF-1 transcriptional activity (13). We show that ACF binds to the PAS-B subdomain of HIF-1α or HIF-2α, thereby blocking het-
cytokines that are critical for tumor vascularization through mobilization of CACs bearing the cognate receptors VEGFR2, CXCR4, and CD117, respectively. ACF treatment decreased tumor-induced CAC mobilization and vascularization, providing a mechanism for tumor growth arrest. ACF inhibited HIF-1 at concentrations that do not affect cell proliferation or survival ex vivo. However, the drug concentrations achieved in vivo were not determined and other mechanisms of action besides HIF-1 inhibition, such as NF-κB inhibition (16), may contribute to the anticancer effects of ACF.

ACF is a mixture of 3,6-diamo-10-methylacridinium chloride (trypanflavin) and 3,6-diamoacridinium (proflavine) (SI Appendix, Fig. S16). Our results provide proof-of-principle that small molecule inhibitors of HIF-1 dimerization in vivo can be identified. Unlike other drugs that indirectly inhibit HIF-1 activity, ACF binds directly to both HIF-1α and HIF-2α. ACF has been administered to patients for at least 5 months without major side effects (14), suggesting that it may be a candidate for clinical trials. ACF can also serve as the lead compound for development of drugs to treat patients with cancer subtypes in which increased HIF-1α or HIF-2α levels are associated with disease progression and patient mortality (2, 5).

Materials and Methods
Detailed materials and methods are available online in SI Appendix.

**Split RLuc System.** DNAs encoding HIF-1α residues 12–396 and HIF-1β residues 11–510 were prepared by PCR (SI Appendix, Table S1) and cloned downstream of the RLuc N-terminal region (residues 1–229) in pCMV-Neu or upstream of the RLuc C-terminal region (residues 230–311) in pCMV-Cruc, respectively (7). HEK293 and HeLa cells were cotransfected with 300 ng of RLuc-HIF-1α12–396, 300 ng of HIF-1α11–510–Cruc, and 80 ng of pGL2-promoter, using FuGene-6 (Roche). After 7-h incubation, cells were treated with drug (9) or vehicle (0.1% DMSO) for 24 h.

**Quantitative Real Time-Reverse Transcription-PCR Assay.** Primers (SI Appendix, Table S2) were designed using Beacon Designer software (Bio-Rad).

**Preparation of His-tagged and GST Fusion Proteins.** HIF-1α12–395 and HIF-2α3–351 were amplified by using specific primers (SI Appendix, Table S3) and PCR products were cloned into pET-28c (Novagen). HIF-1α, HIF-1β, and HIF-2α residues were amplified using specific primers (SI Appendix, Table S4) and the PCR products were inserted into pGEX-SX-1 (GE Healthcare).

**Chromatin IP (ChIP) Assay.** The ChIP Assay Kit (Upstate-Cell Signaling Solution) was used with rabbit polyclonal anti-HIF-1α or HIF-2α Ab (Novus Biologicals) or rabbit IgG. VEGF and PDK1 sequences were detected by PCR (SI Appendix, Table S5).

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Supplemental Materials and Methods

Cell Culture. HEK293, HeLa, and Hep3B-c1 cells were maintained in DMEM; PC-3 and P493 cells were cultured in RPMI (Mediatech); both were supplemented with 10% fetal calf serum (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were grown in a 37°C incubator with 5% CO₂. For hypoxia treatment, cells were placed in a modular incubator chamber (Billups-Rothenberg Inc.) and flushed with a mixture of gas consisting of 1% O₂, 5% CO₂, and balance N₂. The chamber was then sealed and incubated at 37°C. ACF (CAS 8063-24-9) was purchased from Sigma-Aldrich and dissolved in dimethylsulfoxide (DMSO) prior to use.

Split Rluc System and Drug Screening, pCMV-NRluc and pCMV-CRluc plasmids were obtained from S. Gambhir (Stanford University). The human bHLH-PAS domain of HIF-1α (amino acid residues 12-396) was prepared by PCR amplification using primers shown in Table S1. The PCR product was cloned downstream of the N-terminal segment (residues 1-229) of Rluc, in pCMV-NRluc-HIF-1α12-396. Similarly, the bHLH-PAS domain of HIF-1β (residues 11-510) was amplified using primers shown in Table S1 and inserted upstream of the C-terminal segment (residues 230-311) of Rluc, in pCMV-HIF-1β11-510-CRluc.

HEK293 and HeLa cells were seeded at 2x10⁵ cells per well of a 24-well plate and incubated for 20 h. Cells were co-transfected with 300 ng of NRluc-HIF-1α12-396, 300 ng of HIF-1β11-510-CRluc, and 80 ng of pGL2-promoter, using Fugene-6 (Roche) according to the manufacturer's instructions. Following 7 h incubation, cells were treated with drugs from an established library (Zhang et al., 2008) or vehicle (0.1% DMSO) for
24 h. Cells were then lysed and analyzed for the ratio of Rluc/Fluc using the Dual Luciferase Assay System (Promega) and a Victor3 Microplate Reader (PerkinElmer).

**HIF-1-dependent Firefly Luciferase Assay.** HEK293 cells were seeded at 5 x 10⁴ per well of a 24-well plate, incubated for 24 h, and co-transfected with reporter plasmids p2.1, which contains a 68-bp hypoxia response element from the human ENO1 gene upstream of a basal SV40 promoter and firefly luciferase coding sequences, and pSV-Renilla (Zhang et al., 2008). After 24 h, cells were treated with ACF or vehicle, exposed to 20% or 1% O₂ for 24 h, lysed, and analyzed for the ratio of firefly:Renilla luciferase activity.

**Quantitative Real Time-Reverse Transcription-PCR Assay.** Total RNA was extracted using TRIzol (Invitrogen) and treated with DNase (Ambion). A 1-μg aliquot of total RNA was reverse-transcribed using iScript cDNA Synthesis system and qRT-PCR was performed using iQ SYBR Green Supermix and iCycler Real-time PCR Detection System (Bio-Rad). Primers for VEGF, GLUT1, HK1, HK2, SCF, and SDF1 mRNA and 18S rRNA (Table S2) were designed using Beacon Designer software (Bio-Rad) and determined to be specific by BLAST and dissociation curve analysis. The expression level of each mRNA was normalized to the expression of 18S rRNA in the same sample.

**Immunoblot Assays.** Preparation of WCLs and IB analysis were performed as described (Zhang et al., 2008) using antibodies against HIF-1α (Zhong et al., 1999), HIF-2α (Novus Biologicals, Inc.), Flag (Sigma), and β-actin (Santa Cruz Biotechnology).
**Co-immunoprecipitation Assays.** HEK293 cells were exposed to 20 or 1% O₂ in the presence of 5 μM ACF or vehicle for 24 h. WCLs were immunoprecipitated with anti-HIF-1α or –HIF-2α antibody. The WCLs and IP products were subjected to IB assay to detect HIF-1β, HIF-1α, HIF-2α, and β-actin.

**Preparation of Glutathione-S-transferase (GST) Fusion Proteins.** HIF-1β residues 11-510, HIF-1α residues 1-80, 3-155, 3-350, 90-155, 90-350, and 235-350, and HIF-2α (NM_001430) residues 3-72, 3-150, 3-351, 93-150, 93-351, and 237-351 was amplified by PCR using specific primers (Table S4). The PCR products were inserted into pGEX-5X-1 (GE Healthcare). GST fusion proteins were purified as described previously (Baek et al., 2007).

**Preparation of His-tagged Proteins.** HIF-1α12-395 and HIF-2α3-351 was amplified by PCR using specific primers (Table S3). PCR products were cloned into pET-28c (Novagen). *E. coli* BL21 Gold DE3 cells (Stratagene) were transformed with HIF-1α12-395 or HIF-2α3-351 plasmid and treated with 0.5 mM isopropyl-D-thiogalactoside for 6 h. Cells were lysed with MHW buffer (20 mM Tris [pH 7.4], 10 mM imidazol, 0.4 M NaCl, 20% glycerol, 1% Triton X-100, 1 mM DTT, 1 mM Na₃VO₄, 10 mM NaF, and protease inhibitor cocktail). The His-fusion proteins were captured on Ni-NTA agarose beads (Qiagen). After washing with buffer 1 (20 mM Tris [pH 7.4], 0.5 M NaCl, 0.1% Triton X-100, 20 mM imidazol, 10% glycerol) and buffer 2 (20 mM Tris [pH 7.4], 0.1 M NaCl, 10% glycerol), proteins were eluted from beads using Elution buffer (40 mM Tris [pH 7.4], 0.1 M NaCl, 0.1% Triton X-100, 0.2 M EDTA, 20 mM DTT).
**GST Pull Down Assay.** WCLs were prepared from HEK293 cells transfected with pFlag-HIF-1αDM using lysis buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM Na₃VO₄, 10 mM NaF, and protease inhibitor cocktail). An 800-µg aliquot of WCL and 4 µg of GST-HIF-1β₁₁-₅₁₀ or GST were incubated in the presence of ACF or vehicle overnight at 4°C. A 30-µl aliquot of glutathione-Sepharose-4B beads (GE HealthCare) was added to the samples and incubated for 3 h at 4°C. After washing with lysis buffer 3 times, proteins were eluted from beads using Laemmlli buffer and subjected to IB assays.

**In Vitro Dimerization Assay.** His-HIF-1α₁₂-₃₉₅ or His-HIF-2α₃-₃₅₁ was incubated with GST-HIF-1β₁₁-₅₁₀ or GST in the presence of ACF or vehicle overnight at 4°C. GST pull down was performed as described above.

**Fluorometric Analysis.** Purified GST fusion proteins were incubated with ACF or vehicle in lysis buffer overnight at 4°C. The binding complex was captured using GST pull down assay as described above. After washing with lysis buffer 3 times, proteins were hydrolyzed by boiling with elution buffer (50 mM Tris [pH 8.6], and 10 mM reduced glutathione) for 10 min. Soluble ACF was collected by centrifugation (10 min at 13,000 rpm) and analyzed for fluorescence using a SpectraMax M2 microplate reader (Molecular Devices).

**Chromatin IP (ChIP) Assay.** HEK293 cells were plated at 1 x 10⁶ cells per 100-mm dish and incubated for 40 h. Cells were then treated with 5 µM ACF and exposed to 20% or 1% O₂ for 24 h. ChIP was performed with the ChIP Assay Kit (Upstate-Cell Signaling Solution) using rabbit polyclonal anti-HIF-1α or HIF-2α antibodies or normal rabbit IgG
(Santa Cruz). *VEGF* promoter and *PDK1* promoter sequences were detected by PCR using primers listed in Table S5.

**Xenograft Assays.** Protocols were approved by The Johns Hopkins University Animal Care and Use Committee. Male SCID mice were purchased at 5 weeks of age from the National Cancer Institute. Mice were implanted subcutaneously with 5 x 10⁶ PC-3 or Hep3B-c1 cells suspended in 0.2 ml of RPMI or DMEM media, respectively. Tumor volume \( V \) was calculated according to the following formula: \( V = \text{length} \times \text{width} \times \text{height} \times 0.52 \). When tumor volume reached \( \sim 100 \text{ mm}^3 \), mice were divided randomly into treatment and control groups, and ACF or saline was administered, respectively, by daily intraperitoneal injection.

**Flow Cytometry.** Mouse peripheral blood samples were collected and a 100-μl aliquot of anti-coagulated blood was incubated with 1 μg of fluorescein isothiocyanate- and phycoerythrin- conjugated monoclonal antibodies against CXCR4 and Sca1, VEGFR2 and CD34, or VEGFR2 and CD117, respectively (BD Biosciences). Red blood cells were lysed using 0.8% NH₄Cl, 10 mM EDTA (Stem Cell Technologies). Cells were collected by centrifugation 400 x \( g \) and analyzed using a FACScan® flow cytometer (BD Biosciences) that was equipped with an argon laser emitting at 488 nm. Between 60,000 and 100,000 events were collected and the percentage of cells showing dual fluorescence was determined.

**Immunohistochemistry.** Tumor xenografts were fixed in 10% formalin, paraffin-embedded, sectioned into 5-μm slices, mounted on positively charged slides, hydrated through xylene and graded ethanol, and washed with PBS. Endogenous peroxidase activity was quenched using 3% \( \text{H}_2\text{O}_2 \) in methanol and non-specific binding was blocked
by incubation with blocking solution (BioGenex, San Ramon, CA). CD31 (1:50 dilution, BD Pharmigen) or SMA (1:50, Sigma) antibodies were applied to the section for 1 h. Slides were washed with 0.1% Tween-20 in PBS and incubated with biotinylated secondary antibody (1:1000, Vector Laboratory) for 1 h. Immune reactivity was visualized with Vectastain Elite ABC immunoperoxidase system (Vector Laboratory). Sections were counterstained with hematoxylin, dehydrated through graded ethanol into xylene, and mounted. The area of CD31 or SMA staining was quantified (40x objective, 20 fields from each slide) by position pixel algorithm using ImageJ software (NIH).

**ELISA.** Blood samples were allowed to clot and centrifuged at 2,000 g to collect serum. The SDF-1α concentration was determined in duplicate for each serum sample using an ELISA kit (R&D Systems). Optical density was measured at 450 nm and 570 nm (correction) using an AD340 plate reader (Beckman Coulter Inc.). SDF-1α concentration was calculated by linear regression from a standard curve.
Table S1. Primer used for split Rluc-HIF-1 constructs

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Table S2. Primers used for quantitative real-time reverse-transcription PCR

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| HK1   | Fwd: TGGAGTCCAGAGTTTTATG  
          Rev: TTTGGATTGTGGCAAGG  |
| HK2   | Fwd: CGGGCCAAGAGTGCTAAA  
          Rev: TGACGATACCAGGAGCCAATG  |
| SDF1  | Fwd: CTCTGGCAACATGGCTTTCG  
          Rev: GGCTTGAAGATGTACTCG  |
| SCF   | Fwd: AAAGATCCAGAGTCAGCTGTC  
          Rev: TTCCAGTATAAGCTCAAAG  |
| 18s rRNA | Fwd: CGGGCCAAGAGTGCTAAA  
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Table S3. Primers used for His-fusion proteins

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| HIF-2α3-351 | Fwd: CGCGGAATCCCGGAAGATAGTTCTGAACGTCG  
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### Table S4. Primers used for GST-fusion proteins

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### Table S5. Primers used for chromatin immunoprecipitation assay

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Supplemental Figure Legends

**Fig. S1.** Specificity of the split Rluc assay. HEK293 cells were co-transfected with Fluc vector pGL2-promoter and vectors encoding: NRluc-HIF-1α12-396 (NR-1α; 0.3 µg), HIF-1β11-510-CRluc (1β-CR; 0.3 µg), and NRluc-HIF-1β11-510 (NR-1β, A) or double mutant HIF-1α (HIF-1αDM, B). After 36-h incubation, the Rluc/Fluc ratio was determined and each value was normalized to the results for EV. Mean ± SEM is plotted (n = 6). *, P < 0.05; **, P < 0.01 (Student’s t test).

**Fig. S2.** ACF inhibits heterodimerization of HIF-2α and HIF-1β. A, the effect of ACF on HIF-2α:HIF-1β interaction was determined by co-immunoprecipitation (co-IP). HEK293 cells were treated with 5 µM ACF (+) or vehicle (-) and exposed to 20% or 1% O₂ for 24 h. IP of whole cell lysates (WCL) was performed with anti-HIF-2α antibodies (Ab). WCL (left panels) and IP products (right panels) were assayed by immunoblot (IB) to detect HIF-1β, HIF-2α, and β-actin. B, dimerization of HIF-2α and HIF-1β was determined using GST pull down assay. Purified GST-HIF-1β11-510 fusion protein was incubated with WCL obtained from HEK293 cells transfected with expression vector encoding HIF-2α in the presence (+) or absence (-) of 5 µM ACF. Proteins were pulled down with glutathione-Sepharose-4B beads and subjected to IB assays using anti-HIF-2α and anti-GST Ab. An aliquot of WCL was also analyzed directly by IB assay (Input). C, the effect of ACF on HIF-2 dimerization was determined using an *in vitro* binding assay. Purified His-HIF-2α3-351 was incubated with purified GST-HIF-1β11-510 or GST in the presence of ACF or vehicle overnight at 4°C. Binding was determined by GST pull down assay and IB using anti-His and anti-GST Ab.
An aliquot of His-fusion protein was also analyzed directly by IB assay (Input). D, dose-dependent effects of ACF on HIF-2 dimerization in vitro were determined using ACF in the range of 0 to 5 μM. An aliquot of purified His-fusion protein was also analyzed directly by IB assay (Input).

**Fig. S3.** Fluorometric analysis of ACF. The innate fluorescence intensity (FI) of ACF at concentrations ranging from 0 to 1000 μM was determined at 490 nm (emission wavelength) using an excitation wavelength of 463 nm.

**Fig. S4.** ACF binds to the PAS-B domain of HIF-1α and HIF-2α. A, GST-HIF-1α fusion proteins were generated that contained the following HIF-1α amino acid residues: 1-80, 3-155, 3-350, 90-155, 90-350, and 235-350 (top panel). Purified proteins were analyzed by SDS-PAGE and Coomassie blue staining. B, GST (black) and GST fusion proteins containing the following HIF-1α residues were analyzed by FI for binding of ACF (0-250 μM): 1-80 (grey), 3-350 (red), 90-350 (blue), and 235-350 (green). Mean ± SEM is plotted (n = 6) *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs GST (2-way ANOVA with Bonferroni correction). C, GST-HIF-2α fusion proteins were generated that contained the following HIF-2α amino acid residues: 3-72, 3-150, 3-351, 93-150, 93-351, and 237-351. Purified proteins were analyzed by SDS-PAGE and Coomassie blue staining. D, GST (black) and GST fusion proteins containing the following HIF-2α residues were analyzed by FI for binding of ACF (0-250 μM): 3-72 (grey), 3-351 (red), 93-351 (blue), and 237-351 (green). Mean ± SEM is plotted (n = 6) *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs GST (2-way ANOVA with Bonferroni correction).
Fig. S5. Acriflavine (ACF) does not bind to the bHLH-PAS domain of AHR. Purified GST fusion proteins that contained no insert (black), HIF-1α_{235-350} (grey), HIF-2α_{237-351} (red), AHR_{1-410} (blue), or AHR_{281-410} (green) were analyzed by SDS-PAGE and Coomassie blue staining (left panel). Each fusion protein was incubated with ACF, captured with glutathione beads, washed, and analyzed for fluorescence intensity (FI) (right panel). Mean ± SEM is plotted (n = 4) *, P < 0.05; ***, P < 0.001 vs GST (2-way ANOVA with Bonferroni correction).

Fig. S6. ACF does not induce homodimerization of HIF-1α. A, the effect of ACF on HIF-1α:HIF-1α homodimerization was determined using the GST pull-down assay. Purified GST, GST-HIF-1α_{2-350}, or GST-HIF-1β_{11-510} was incubated with lysates prepared from hypoxic HEK293 cells in the presence of 5 μM ACF (+) or vehicle (-). Proteins were pulled down with glutathione-Sepharose-4B beads and subjected to immunoblot (IB) assay using anti-HIF-1α and anti-GST antibodies. B-C, homodimerization of HIF-1α was investigated by in vitro binding assays. Purified His-HIF-1α_{12-395} was incubated with GST, GST-HIF-1α_{2-350}, or GST-HIF-1β_{11-510} in the presence of 5 μM ACF (+) or vehicle (-). The proteins bound to Ni-NTA agarose were assayed by IB to detect GST- and His-fusion proteins (B). Binding was also determined by GST pull-down assay and IB using anti-His and anti-GST antibodies (C).

Fig. S7. ACF inhibits HIF-2 DNA-binding activity. HIF-2 DNA-binding activity was determined by chromatin IP assay. HEK293 cells were exposed to 20% or 1% O₂ in the presence of 5 μM ACF or vehicle control (Con). Input DNA was isolated from an aliquot of lysate before IP. The remaining lysate was divided in half and incubated with anti-HIF-2α
antibodies or normal rabbit IgG for IP. PCR was performed using the immunoprecipitates as template to amplify sequences from the VEGF promoter (upper panels) and PDK1 promoter (bottom panels), which contain known HIF-1 binding sites. PCR products were fractionated by 2% agarose gel electrophoresis and analyzed by ethidium bromide staining. M, 200-bp size marker.

**Fig. S8.** Effects of ACF on HIF-1α:Hsp90 interaction and MYC-regulated gene expression. 
A, the effect of ACF on the association of HIF-1α and Hsp90 was determined using co-IP. HEK293 cells were exposed to 20% or 1% O2 in the presence of 5 µM ACF (+) or vehicle (-) for 24 h. IP of WCL was performed with anti-HIF-1α antibody. WCL and IP products were assayed by IB to determine the levels of Hsp90, HIF-1α, and β-actin protein. B, effect of ACF on the expression of MYC-target genes was determined by quantitative real-time reverse-transcription PCR (qRT-PCR). Human P493 cells that conditionally repress MYC upon exposure of tetracycline were treated with tetracycline or vehicle for 48 h. During the last 24 h, cells were exposed to 20% or 1% O2 in the presence of 5 µM ACF (black bars) or vehicle (white bars). The expression levels of fibrillarin (FBL), apurinic/apyrimidinic exonuclease (APEX), and GLUT1 mRNA were determined. Target mRNA levels relative to 18S rRNA in vehicle- and ACF-treated cells were calculated as 2^ΔΔCt, where ΔCt = Ct_target - Ct_18S and Δ(ΔCt) = ΔCt_vehicle - ΔCt_ACF. Mean ± SEM is plotted (n = 6). **, P < 0.01 vs 20%, vehicle-treated, myc-off; ††, P < 0.01 vs 1%, vehicle-treated, myc-off (2-way ANOVA).

**Fig. S9.** Effect of ACF on HIF-1 activity in cultured PC-3 cells. A, HIF-1α protein levels were determined by IB assay with WCL prepared from PC-3 cells that were exposed to
hypoxia (1% O_2, 20 h) in the presence of vehicle control (Con) or 1-10 μM ACF. B, the effect of ACF on HIF-1-dependent firefly luciferase (Fluc) activity in PC-3 cells was determined. PC-3 cells were co-transfected with HIF-1-dependent Fluc reporter plasmid p2.1 and Rluc reporter plasmid pSV-Renilla. After 24-h incubation, cells were treated with ACF at concentrations ranging from 0 to 5 μM and exposed to 20% (white bars) or 1% (black bars) O_2 for 24 h. Cells were then lysed and analyzed for the ratio of Fluc:Rluc activity. Mean ± SEM is plotted (n = 6). *, P < 0.05; **, P < 0.01 (2-way ANOVA). C, VEGF, SDF1, SCF, GLUT1, HIF-1α, HIF-2α, and RPL13A mRNA levels were determined using qRT-PCR in PC-3 cells treated with vehicle (Con) or 5 μM ACF under 20% (white bars) or 1% (black bars) O_2. Target mRNA levels relative to 18S rRNA in tumors from vehicle- and ACF-treated mice were calculated as 2^-\Delta(\Delta Ct), where ΔCt = Ct_{target} - Ct_{18S} and Δ(ΔCt) = ΔCt_{vehicle} - ΔCt_{ACF}. Mean ± SD (n = 4) are shown. **P < 0.01 vs Con (2-way ANOVA).

Fig. S10. Effects of ACF on the expression of proteins in PC-3 and Hep3B-c1 cells. PC-3 and Hep3B-c1 cells were exposed to 20% or 1% O_2 in the presence of 5 μM ACF (+) or vehicle (-) for 24 h. WCL were analyzed to determine HIF-1α, HIF-2α, HIF-1β, c-Myc, Hsp90, and β-actin protein levels by IB assays.

Fig. S11. Effect of ACF on PC-3 prostate cancer cell growth in tissue culture. A, PC-3 cells were cultured at 20% or 1% O_2 in the presence of vehicle or 5 μM ACF for 0-72 h and the number of viable cells was determined by trypan blue staining. B, PC-3 cells were cultured for 72 h at 20% or 1% O_2 in the presence of vehicle or 5 μM ACF, stained with propidium iodide, and subjected to flow cytometry to determine the percentage of cells at each cell cycle stage. Mean ± SEM (n = 3) values are shown. C-D, P493 (C) and Hep3B-
c1 (D) cells were cultured at 20% or 1% O₂ in the presence of vehicle or 5 µM ACF for 0-72 h and the number of viable cells was determined by trypan blue staining.

**Fig. S12.** ACF pretreatment prevents human prostate tumor xenograft growth. 5 x 10⁶ PC-3 cells were implanted into subcutaneous tissue of SCID mice receiving daily intraperitoneal injections of vehicle (black) or 2 mg/kg ACF (red), starting 3 d prior to implantation, and tumor volume was measured. Mean ± SEM (n = 4) is shown. *, P < 0.05; **, P < 0.01 vs vehicle (2-way ANOVA).

**Fig. S13.** Body weights of tumor-bearing mice treated with vehicle or ACF. A, weights of the tumor-bearing mice from Fig. S12 that were treated with vehicle (black) or ACF (red) were measured. Mean ± SEM (n = 4) is shown. B, weights of the tumor-bearing mice from Fig. 6A that were treated with vehicle (black) or ACF (1 mg/kg, blue; 2 mg/kg, red) were measured. Mean ± SEM (n = 4) is shown. C, weights of the tumor-bearing mice from Fig. 6C that were treated with vehicle (black) or ACF (2 mg/kg, red) were measured. Mean ± SEM (n = 8) is shown. D, weights of the tumor-bearing mice from Fig. 7 and S15 that were treated with vehicle (black) or ACF (2 mg/kg, red) were measured. Mean ± SEM (n = 8) is shown.

**Fig. S14.** ACF does not affect HIF-1α protein expression in human tumor xenografts. Lysates of tumor tissues from the xenograft experiment shown in Fig. 6A were subjected to IB assays of HIF-1α and β-actin protein levels.
**Fig. S15.** Effect of short-term ACF treatment on prostate tumor growth. PC-3 xenografts were grown to a mean volume of 100 mm$^3$ and the mice were treated daily for 9 d with vehicle (black) or ACF (2 mg/kg, red). The plotted tumor volume data represent mean ± SEM ($n = 8$). **, $P < 0.01$ vs vehicle-treated tumor-bearing mice (2-way ANOVA). These mice were further analyzed in Fig. 7.

**Fig. S16.** ACF is a mixture of proflavine and trypaflavin, the chemical structures of which are shown.
Fig. S1

A

![Bar graph showing the effect of NR-1α + 1β-CR on Rluc/Fluc ratio.](image)

B

![Bar graph showing the effect of HIF-1αDM on Rluc/Fluc ratio.](image)
Fig. S6

A

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ACF (5 µM)

HIF1α

GST-HIF1β

GST-HIF1α

GST

B

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ACF (5 µM)

GST-HIF1β

GST-HIF1α

GST

His-HIF1α

C

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ACF (5 µM)

His-HIF1α

GST-HIF1β

GST-HIF1α

GST
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**VEGF promoter**

**PDK1 promoter**
Fig. S8

A

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B

[Graphs showing mRNA levels for FBL, APEX, and GLUT1 under different conditions]
Fig. S9

Panel A: Western blot analysis of HIF1α and β-Actin under 20% O2 and 1% O2 conditions.

Panel B: Bar graph showing Fluc/Rluc ratio at different concentrations of ACF under 20% O2 and 1% O2 conditions. Significant differences are indicated with * and **.

Panel C: Bar graphs showing mRNA expression levels of VEGF, SDF1, SCF, GLUT1, HIF1α, and HIF2α under 20% O2 and 1% O2 conditions. Significant differences are indicated with **.
**Fig. S10**

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Fig. S14

ACF

Vehicle 1 mg/kg 2 mg/kg

HIF-1α

β-Actin
Fig. S15
Acriflavine

Proflavine

Trypaflavin