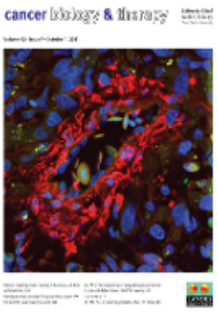

Cancer Biology & Therapy

ISSN: 1538-4047 (Print) 1555-8576 (Online) Journal homepage: https://www.tandfonline.com/loi/kcbt20

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To cite this article: Jolanta Paradziej-Łukowicz, Anna Skwarska, Grażyna Peszyńska-Sularz, Anna Brillowska-Dąbrowska & Jerzy Konopa (2011) Anticancer imidazoacridinone C-1311 inhibits hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF) and angiogenesis, Cancer Biology & Therapy, 12:7, 586-597, DOI: 10.4161/cbt.12.7.15980

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Anticancer imidazoacridinone C-1311 inhibits hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF) and angiogenesis

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Keywords: chemotherapy, imidazoacridinone C-1311, hypoxia, HIF-1, VEGF, angiogenesis

Abbreviations: bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; C-1311, 5-diethylaminoethylamino-8-hydroxyimidazoacridinone; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HIF-1α, hypoxia-inducible factor-1alpha; HRE, hypoxia-responsive element; i.p., intraperitoneal; MEM, minimal essential medium; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; s.c., subcutaneously; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor

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Antitumor imidazoacridinone C-1311 is a DNA-reactive topoisomerase II and FLT3 receptor tyrosine kinase inhibitor. Here, we demonstrate the mechanism of C-1311 inhibitory action on novel targets: hypoxia-inducible factor-1α (HIF-1α), vascular-endothelial growth factor (VEGF), and angiogenesis. In a cell-free system, C-1311 prevented HIF-1α binding to an oligonucleotide encompassing a canonical hypoxia-responsive element (HRE), but did not directly interfere with HIF-1α protein. Whereas C-1311 did not affect HIF-1α transcription, at higher concentrations (10 μM), it decreased HIF-1α protein accumulation. Furthermore, C-1311 potently reduced the transcription of HIF-1-dependent reporter gene as well as endogenous HIF-1 target gene encoding vascular endothelial growth factor. In colon cancer HCT116 and murine melanoma B16/F10 cells, C-1311-induced downregulation of *VEGF*, resulted in decreased VEGF protein expression. C-1311 did not alter normoxic VEGF secretion. Consistent with VEGF depletion, C-1311 significantly affected angiogenesis in vivo. A single dose of C-1311 (40 mg/kg), inhibited angiogenesis by 70%, as measured by vascularization of Matrigel plugs in mice. Continuous low C-1311 dosing (8 mg/kg/day for 5 d) gave similar inhibition of angiogenesis (80%), suggesting that low-dose, frequent C-1311 administration may be more effective in terms of reducing toxicity. Anti-angiogenic activity of C-1311 was further demonstrated in an experimental model in which B16/F10 cells were encapsulated in alginate beads and implanted into mice. C-1311 (8 mg/kg/day for 9 d), completely abrogated vascularization of the alginate implants. Our findings indicate that C-1311 is an effective inhibitor of HIF-1α, VEGF and angiogenesis and provide new perspectives into the mechanism of its anticancer activity.

Introduction

The imidazoacridinones are a new class of antitumor agents rationally designed based on structure-activity relationship studies of the chemotherapeutic agent mitoxantrone.[1] So far, the most promising imidazoacridinone derivative, C-1311 (Symadex™; **Fig. 1A**), was evaluated in Phase II clinical trials where it displayed significant activity toward breast cancers.[2,3] C-1311 exerts its toxic effect primarily by causing DNA damage. The drug binds to DNA non-covalently (by intercalation)[4,5] and covalently, following oxidative metabolic activation.[6] The covalent binding is likely to underlie the demonstrated ability of C-1311 to form interstrand crosslinks in cellular DNA.[7,8] In addition, C-1311 inhibits the catalytic activity of topoisomerase II and traps the cleavable complexes between DNA and topoisomerase II, in cell-free systems and in living cells.[9] Our previous studies demonstrated that, in response to C-1311-induced DNA damage, human MOLT4 leukemia cells underwent transient G_2 phase arrest, followed by

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Submitted: 02/20/11; Revised: 06/20/11; Accepted: 06/22/11
DOI:10.4161/cbt.12.7.15980

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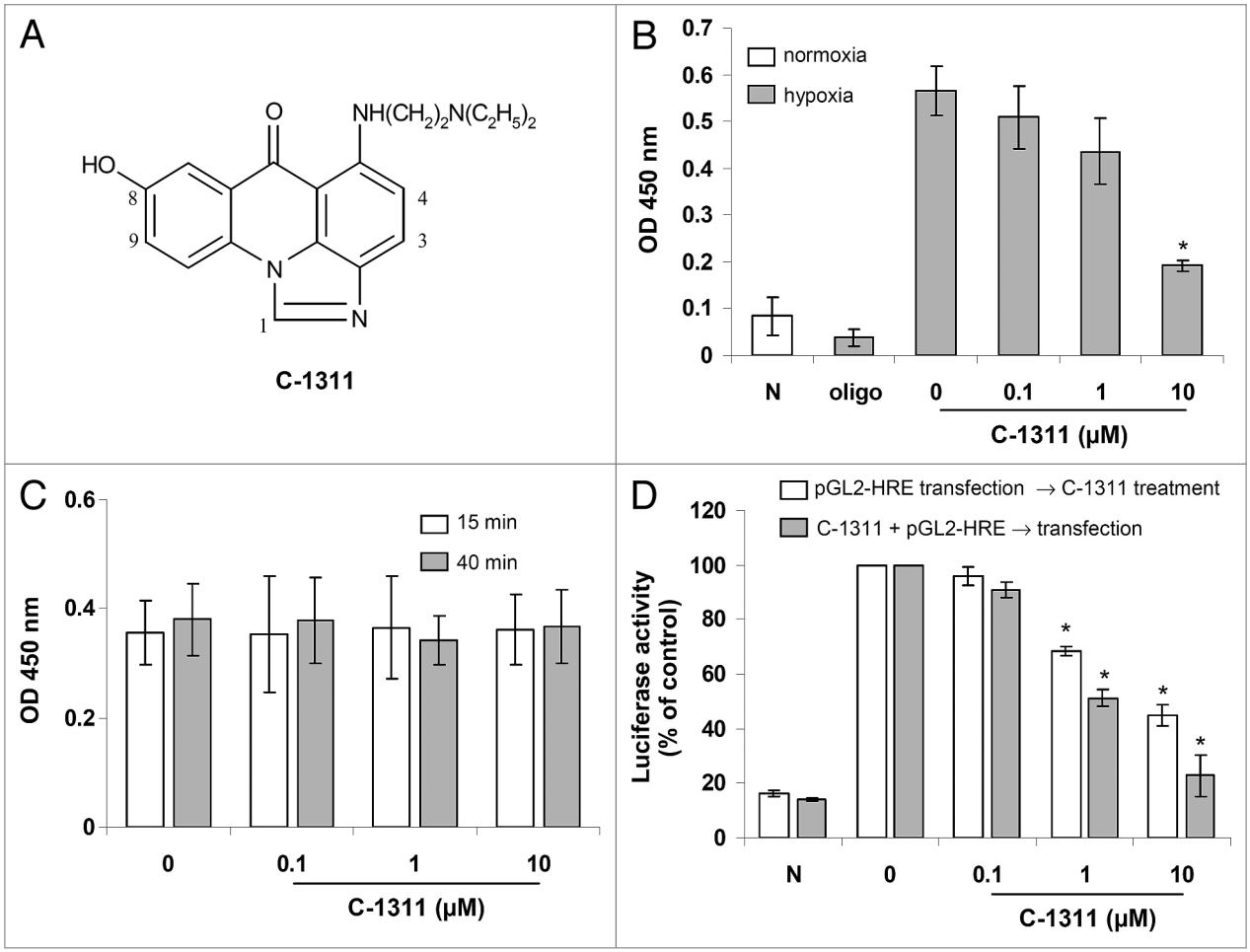

Figure 1. Imidazoacridinone C-1311 downregulates HIF-1 transcriptional activity by blocking HIF-1α binding to HRE. (A) Chemical structure of C-1311. (B) C-1311, at indicated concentrations, was added to HRE double-strand DNA probe directly prior to the addition of HIF-1α-rich nuclear extracts from HCT116 cells. HIF-1α binding to the HRE sequence was determined by ELISA. Nuclear extracts from normoxic HCT116 cells (*N*) and hypoxic nuclear extracts incubated with a HIF-1α-specific competitor oligonucleotide (*H⁺oligo*) served as negative and positive controls (C) C-1311 was directly incubated with nuclear extracts from hypoxic HCT116 cells for 15 or 40 min. After preincubation, C-1311 and nuclear extract mix was added to DNA HRE probe. HIF-1α binding to HRE sequence was determined by ELISA. (D) HCT116 cells were transiently transfected with a pGL2-HRE plasmid expressing luciferase under the control of HRE. After transfection, cells were treated with C-1311 under hypoxia for 24 h and analyzed for luciferase luminescence assay. Alternatively, HRE reporter plasmid was preincubated with C-1311 for 30 min before cell transfection and cells were subjected to luciferase assay 24 after transfer. Luminescence values were normalized to β-galactosidase expression and presented as percentage of control value (untreated cells under hypoxia). Results are the mean ± SE of three independent experiments; *$p < 0.01$ between the indicated concentrations and hypoxia control.

mitotic catastrophe, which in turn precipitated delayed, yet massive apoptosis.[10] The anti-proliferative and apoptosis-inducing effects of C-1311 have also been documented in other cell types including murine leukemia,[11] human myeloid and lymphoblastic leukemia,[12] human ovarian cancer and osteogenic sarcoma cells.[13] C-1311 was also demonstrated to have appreciable cytotoxic activity in human colon,[14] lung, melanoma[15] and bladder cancer cell lines;[16] nevertheless, apoptosis was not the main cell-killing mechanism induced by this drug. More recent studies point to an alternative mechanism of C-1311 action; C-1311 was found to be a potent and selective inhibitor of quinone oxidoreductase NQO2,[17] and receptor tyrosine kinase FLT3.[18] Given that FLT3 kinase is important in the differentiation of progenitors into dendritic cells involved in an autoimmune response,[19] early preclinical tests have already shown promising pharmacological activity of C-1311 in the treatment of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.[20]

Studies from our laboratory have revealed that C-1311, binds to DNA with limited sequence specificity but, following metabolic activation, this compound selectively and covalently binds to guanine (Dziegielewski J and Konopa J, manuscript in preparation). Independently, it was shown that a close analog of C-1311, triazoloacridinone C-1305, selectively intercalates within guanine triplets and induces unusual structural changes that could be important for the cytotoxic activity of this compound.[21] It is well established that many functional DNA regions in the human genome contain guanine-rich sequences, including GC-rich gene promoter regions. Among these is the hypoxia-responsive element (HRE) that contains the core sequence 5'-(A/G)CGTG-3' and is present in promoters of hypoxia-inducible factor-1 (HIF-1)

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target genes.[22] Because even single methylation of guanine residues in HREs reduced HIF-1 binding by 100%, we postulated that imidazoacridinone C-1311, with its preferential covalent binding to guanine, may interfere with the DNA binding site recognized by HIF-1.

Hypoxia-inducible factor-1 (HIF-1), a heterodimer composed of two subunits, HIF-1α and HIF-1β, is a transcription factor expressed in response to oxygen deprivation, commonly developed in rapidly growing solid tumors.[22] HIF-1 activity is dependent upon the level of available HIF-1α, making this component a new target for anticancer therapies.[23] Under normoxic conditions, human HIF-1α is posttranslationally modified by propyl hydroxylases, targeting the protein for proteasomal degradation, and by asparagine hydroxylase, preventing its interaction with transcription coactivators.[24] Limited oxygen availability prevents these modifications, leading to HIF-1α accumulation, and translocation into the nucleus where it forms an active complex with HIF-1β, which specifically binds to target gene promoters containing HRE. HIF-1 heterodimer activates transcription of a wide variety of genes important for adaptation and survival under hypoxia, including genes involved in glucose metabolism, erythropoiesis or resistance to apoptosis.[22] Moreover, HIF-1 is a key transcription factor controlling angiogenesis through its effect on the expression of *VEGF*, which encodes vascular endothelial growth factor.[25] Importantly, HIF-1 loss of function in human cancer cells has been shown to decrease VEGF expression and tumor vascularization.[26] VEGF promotes tumor angiogenesis through several mechanisms, including enhanced endothelial cell proliferation and survival; increased migration and invasion of endothelial cells; increased permeability of existing vessels; and enhanced chemotaxis and homing of bone marrow-derived vascular precursor cells.[27] Inhibiting VEGF expression and/or VEGF receptor signaling—alone or in combination with other treatments—has shown promise for tumor anti-angiogenesis therapy both in animal models and cancer patients.[28]

This study demonstrates for the first time that imidazoacridinone C-1311 inhibits HIF-1 activity. The drug prevents HIF-1α binding to HRE sequence, thus decreasing its transactivating activity and downstream VEGF expression. We also demonstrate the anti-angiogenic effect of C-1311 in vivo, suggesting that this is a consequence of C-1311-driven VEGF inhibition.

Results

Imidazoacridinone C-1311 downregulates HIF-1 transcriptional activity by blocking HIF-1α binding to HRE. To investigate whether imidazoacridinone C-1311 could affect the activity of HIF-1α, we first examined its effect on HIF-1α binding to DNA using an ELISA kit with an HRE double-strand DNA probe covalently bound to the bottom of a multi-well plate. To obtain HIF-1α-rich nuclear extracts, HCT116 human colon cancer cells were subjected to hypoxic conditions for 16 h. The HCT116 cell line was selected due to its high sensitivity to C-1311 in the NCI in vitro screening system. Preincubation of C-1311 with oligonucleotide encompassing the canonical HIF-1α binding site (HRE sequence) for 15 min before addition of hypoxic nuclear extracts resulted in dose-dependent inhibition of HIF-1α DNA-binding activity, with significant inhibition (66%) at 10 μM drug concentration (**Fig. 1B**). Prolonged drug incubation with HRE DNA probe for 40 min almost completely blocked HIF-1α DNA binding to HRE at 10 μM (data not shown). In contrast, direct addition of up to 10 μM C-1311 to hypoxic HCT116 nuclear extracts did not affect HIF-1α binding, regardless of the time of drug exposure (**Fig. 1C**).

We next studied the effect of C-1311 on HIF-1 transcriptional activity using reporter gene assay. HCT116 cells were transiently transfected with pGL2-HRE reporter plasmid containing luciferase cDNA regulated by HRE from *VEGF* promoter.[29] After transfection, cells were exposed to C-1311 for 24 h under hypoxia. As shown in **Figure 1D**, luciferase activity was markedly induced under hypoxia compared with normoxic conditions and this activity was significantly inhibited by increasing doses of C-1311. Direct preincubation of C-1311 with HRE reporter plasmid before cell transfection caused more profound inhibition of HRE activity. At the maximal C-1311 dose (10 μM) there was ~80% significant decrease of HIF-1 transcriptional activity.

Imidazoacridinone C-1311 weakly affects hypoxia-induced HIF-1α expression in human HCT116 cells. ELISA experiments (**Fig. 1B and C**) and gene reporter assay (**Fig. 1D**) showed that C-1311 potently inhibited HIF-1α DNA binding to HRE. It is well known that hypoxia-induced HIF-1 DNA binding and transcriptional activity are dependent on increased levels of HIF-1α protein.[22] To investigate whether C-1311 affects HIF-1 activity by altering expression of HIF-1α, we first examined the effect of C-1311 on the levels of HIF-1α mRNA in hypoxic HCT116 cells. Real-time PCR analysis showed that upon treatment of HCT116 cells with C-1311 for 24 or 48 h under hypoxia, HIF-1α mRNA expression was not significantly ($p > 0.05$) affected by any concentration of the drug (**Fig. 2A**). In untreated cells, HIF-1α levels were relatively decreased under hypoxia compared with normoxia (**Fig. 2A**). Of note, C-1311 did not affect the levels of β-actin mRNA that was used as a normalization control (data not shown).

We next examined nuclear HIF-1α protein expression in HCT116 cells under hypoxia or in the presence of hypoxia mimicking $CoCl_2$, which stabilizes HIF-1α expression in cells under normoxia.[30] As shown in **Figure 2B**, compared with normoxic conditions, hypoxia induced a robust accumulation of HIF-1α protein (on protein gel blots presented as a series of bands from 106 to 116 kDa). Following 24 h treatment, C-1311 at a concentration of 0.1 and 1 μM did not significantly affect the level of HIF-1α protein. A slight decrease of HIF-1α expression was detectable under hypoxia at drug concentration of 10 μM. Similar results were obtained under $CoCl_2$-simulated hypoxia. After prolonged (48 h) incubation in hypoxic conditions, at higher dose of C-1311 (10 μM), the decrease in the level of HIF-1α protein was more evident.

Imidazoacridinone C-1311 inhibits hypoxia-induced VEGF expression in HCT116 cells. Given that HIF-1α is the main regulator of *VEGF* expression and that under hypoxia, *VEGF* transcription requires integrity of the HIF-1α-binding site,[31] we next analyzed whether the inhibition of HIF-1α activity would result

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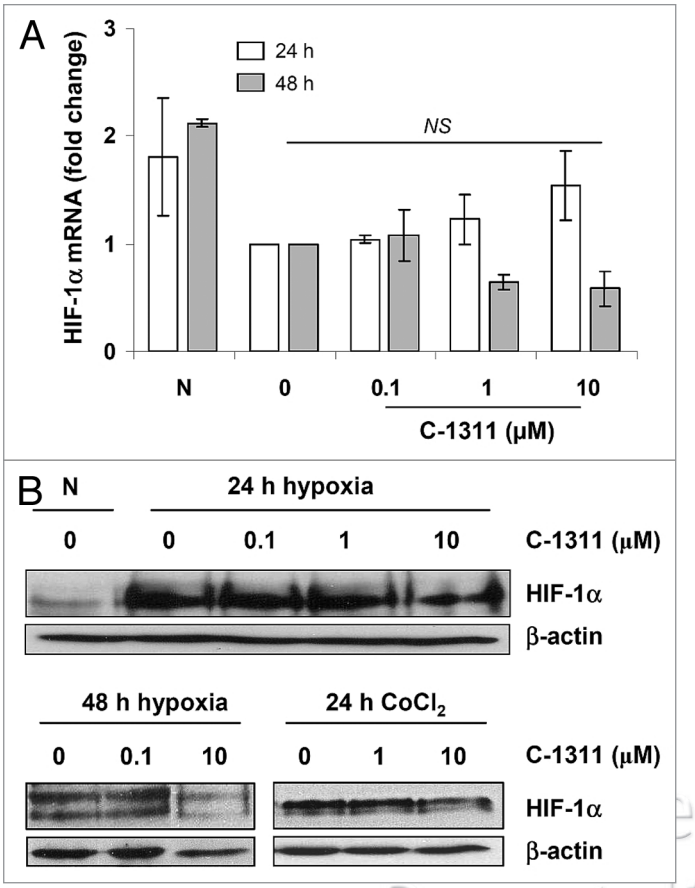

Figure 2. Imidazoacridinone C-1311 weakly affects hypoxia-induced HIF-1α expression in human HCT116 cells. (A) HCT116 cells were left untreated or exposed to C-1311 under hypoxia for the indicated times. HIF-1α mRNA levels were assessed by real-time PCR and normalized to β-actin mRNA levels. Results are expressed as fold change relative to HIF-1α mRNA levels under hypoxic conditions (equal to 1) in the absence of drug. The bars represent the mean ± range of three independent experiments; NS, $p > 0.05$ between the indicated concentrations and hypoxia control. *N*, untreated cells under normoxia. (B) Protein gel blot analysis of HIF-1α protein. HCT116 cells were exposed to C-1311 for 24 and 48 h under hypoxia or treated with C-1311 for 24 h in the presence of $CoCl_2$ (300 μM). Non-treated HCT116 cells under normoxia were used as a negative control (*N*). For each protein gel blot analysis, 50 μg of nuclear extracts were subjected to SDS-PAGE and immunoblotted with antibody against HIF-1α. β-actin was used as a loading control. Similar results were observed in three independent experiments.

in decreased expression of *VEGF*. To address this, HCT116 cells were transfected with reporter plasmid pGL2-VEGF containing luciferase cDNA under control of a full-length VEGF promoter.[29] After transfection, cells were exposed to C-1311 for 24 h under hypoxia. As shown in **Figure 3A**, hypoxia potently induced the VEGF transcription, while C-1311 treatment decreased it by 40 and 52% at 1 and 10 μM. The extend of changes in VEGF promoter activity was more profound upon direct preincubation of pGL2-VEGF vector with C-1311 before cell transfection. In these conditions, ~60 and ~85% inhibition was produced by C-1311 at 1 and 10 μM (**Fig. 3A**).

To further investigate the effect of C-1311 on HIF-1 transcriptional activity, we measured the transcript levels of *VEGF*. HCT116 cells were incubated under hypoxic conditions for 24 and 48 h in the presence or absence of 0.1–10 μM C-1311 and the mRNA expression of VEGF was evaluated by real-time PCR. As shown in **Figure 3B**, the mRNA expression of VEGF was noticeably induced under hypoxia. C-1311 had a significant dose- and time-dependent inhibitory effect on hypoxic induction of VEGF mRNA expression. In particular, following 24 h treatment, C-1311 reduced VEGF mRNA level by 18, 34 and 47% at 0.1, 1 and 10 μM. By 48 h, the VEGF mRNA levels dramatically dropped, with 76% inhibition at 1 μM and 82% inhibition at 10 μM C-1311.

To further assess whether the decreased VEGF mRNA levels affected VEGF protein production, we measured VEGF protein secretion in HCT116 cell-conditioned medium using ELISA. As anticipated, C-1311 inhibited hypoxia-induced VEGF release to the extracellular medium in a dose-and time-dependent manner corresponding to VEGF mRNA downregulation (**Fig. 3C**). After 24 h, ~20 and ~30% inhibition was produced by C-1311 at 1 and 10 μM. The observed effect was more pronounced upon prolonged drug exposure. After 48 h, the inhibition of VEGF secretion reached ~53% in cells cultured with 10 μM C-1311.

Effect of p53 genotype on hypoxia-induced VEGF protein expression following C-1311 treatment. The p53 tumor suppressor protein is induced and activated in response to a variety of cellular stressors including DNA damage and hypoxia.[32] Consistent with the fact that C-1311 is a DNA-damaging agent, protein gel blot analysis showed that the drug induced dose-dependent expression of p53 in HCT116 $p53^{+/+}$ cells under 24 h hypoxia (**Fig. 4A**). Given that p53 protein is a potent downregulator of VEGF,[33] to rule out its negative influence on VEGF expression upon C-1311 treatment, we analyzed the effect of C-1311 on VEGF secretion in p53-deficient HCT116 cells ($p53^{-/-}$). Following exposure to hypoxia for 24 h, $p53^{-/-}$ cells demonstrated slightly lower VEGF protein production (**Fig. 4B**) in comparison to their $p53^{+/+}$ counterparts. C-1311 at 0.1 and 1 μM, when combined with hypoxia, had no effect on the level of VEGF in conditioned medium. In contrast, 10 μM C-1311 resulted in ~30% significant inhibition of VEGF secretion (**Fig. 4B**).

Imidazoacridinone C-1311 does not affect normoxic VEGF expression. Although VEGF is strongly induced by hypoxia, its basal expression is regulated by HIF-1-independent mechanisms.[31] Using cell-based gene reporter assay we found that C-1311 affected normoxic VEGF expression only when the drug was directly preincubated with pGL2-VEGF plasmid before transfection into HCT116 cells (**Fig. 5A**). C-1311 did not significantly change luciferase activity when cells were transfected with pGL2-VEGF vector and subsequently treated with the drug under normoxia.

Real-time PCR analysis of RNA prepared form untreated or treated HCT116 cells with increasing doses of C-1311 under normoxia revealed that also endogenous VEGF mRNA expression was not significantly ($p > 0.05$) affected by any concentration of C-1311 (**Fig. 5B**). Consistent with these observations, there was no significant difference in secretion of VEGF protein into conditioned medium before and after drug treatment in normoxic conditions (**Fig. 5C**).

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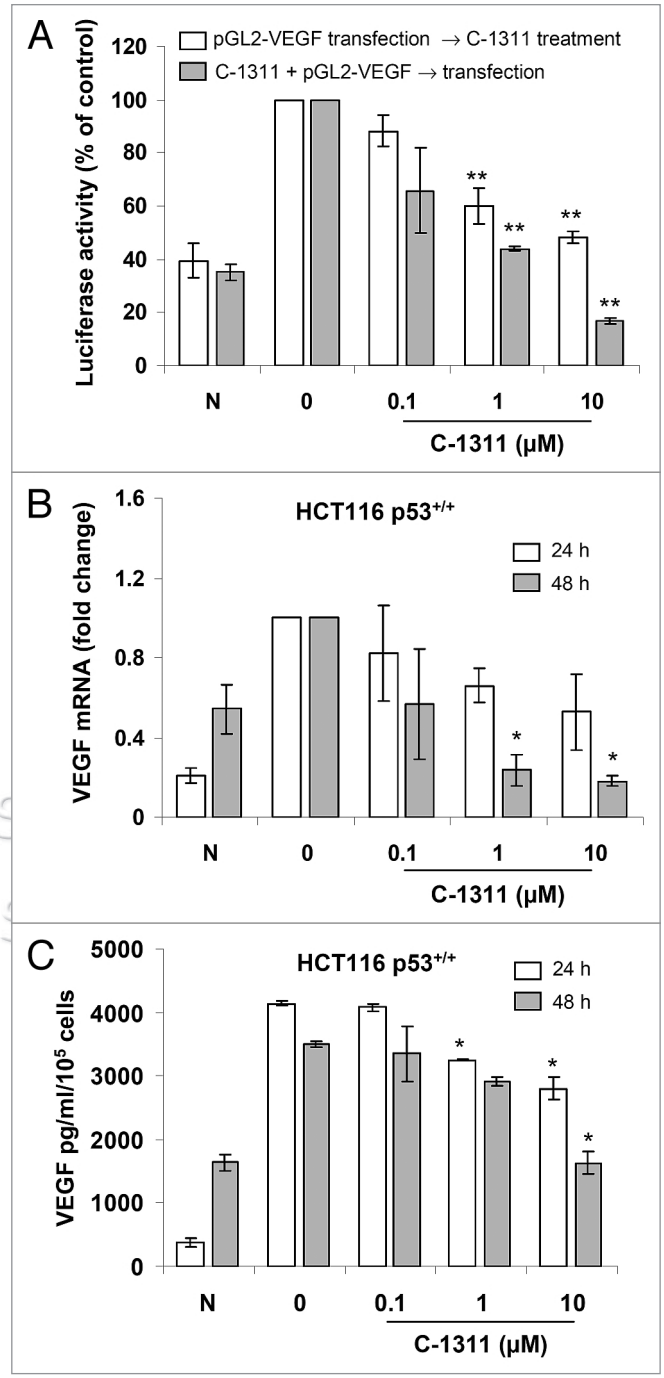
Figure 3. Imidazoacridinone C-1311 inhibits hypoxia-induced VEGF expression. (A) The effect of C-1311 on the full-length VEGF promoter. HCT116 cells were transiently transfected with a pGL2-VEGF plasmid expressing luciferase under the control of a full-length human VEGF promoter. After transfection, cells were treated with C-1311 under hypoxia for 24 h and analyzed for luciferase luminescence assay. Alternatively, pGL2-VEGF reporter plasmid was preincubated with C-1311 for 30 min before cell transfection and cells were subjected to luciferase assay 24 after transfer. Luminescence values were normalized to β-galactosidase expression and presented as percentage of control value (untreated cells under hypoxia). Results are the mean ± SE of three independent experiments; **$p < 0.01$ between the indicated concentrations and hypoxia control. (B) The effect of C-1311 on VEGF mRNA. HCT116 cells were incubated in hypoxic conditions in the presence of C-1311 for 24 and 48 h. VEGF mRNA levels were assessed by real-time PCR and normalized to β-actin mRNA levels. Results are expressed as fold change relative to VEGF mRNA levels under hypoxic conditions (equal to 1) in the absence of drug. The bars represent the mean ± range of three independent experiments; *$p < 0.05$ between the indicated concentrations and hypoxia control. (C) The effect of C-1311 on VEGF secretion. HCT116 cells were incubated in hypoxic conditions in the presence of C-1311 for 24 and 48 h. The extracellular medium was recovered for VEGF determination by ELISA. VEGF level expressed as picogram per milliliter per 10^5 cells. Bars represent mean ± range of three independent experiments; *$p < 0.05$ between the indicated concentrations and hypoxia control. *N*, untreated cells under normoxia.



Imidazoacridinone C-1311 weakly affects HIF-1α accumulation but significantly decreases VEGF protein expression in a mouse melanoma B16/F10 cell model. Our data thus far identified C-1311 as an effective inhibitor of HIF-1α transcriptional activity and VEGF expression in human colon adenocarcinoma HCT116 cells. We next determined whether C-1311 downregulates HIF-1α activity in transplantable murine melanoma B16/F10 cells. We selected these cells due to their utility in the following angiogenesis inhibitory studies in mice. B16/F10 cells were incubated under hypoxic conditions in the presence or absence of 0.1–10 μM C-1311 and the mRNA and protein expression of HIF-1α and VEGF were evaluated. As shown in **Figure 6A** and C-1311 treatment did not significantly ($p > 0.05$) affect HIF-1α on the transcriptional level. Whereas HIF-1α mRNA expression remained relatively unchanged before and after C-1311 treatment under 48 h hypoxia, HIF-1α protein accumulation was inhibited at the maximal C-1311 dose (10 μM) (**Fig. 6B**). Analysis of VEGF expression revealed that the levels of VEGF mRNA (**Fig. 6C**) and protein (**Fig. 6D**) were slightly elevated by treatment with C-1311 in 24 h hypoxia. In contrast, after 48 h, C-1311 induced pronounced (~50%) inhibition of VEGF mRNA expression irrespective of the drug concentration (0.1–10 μM) (**Fig. 6C**), accompanied by a gradual decrease in VEGF protein secretion with ~35% inhibition at 10 μM (**Fig. 6D**).

Imidazoacridinone C-1311 inhibits angiogenesis in the mouse Matrigel plug assay. Further experiments explored the possibility that C-1311 directly inhibits tumor angiogenesis in vivo, as might be expected based on HIF-1α inhibition/depletion and decreased VEGF expression. These experiments were conducted in C57BL/6 mice using the Matrigel plug assay and bFGF as the strong angiogenic stimulus.[34] Hemoglobin content of the Matrigel plugs served as an indicator of neovascularization. Cyclophosphamide, known for its anti-angiogenic properties, was used as a reference drug.[35] As shown in **Figure 7A**, in Matrigel pellets lacking bFGF (negative control), hemoglobin content was minimal relative to that of Matrigel plugs containing bFGF alone. Administration of C-1311 as a single i.p. dose of 40 mg/kg resulted in a drastic reduction of hemoglobin level, indicating decreased blood vessel formation ($p < 0.01$). The calculated degree of angiogenesis inhibition (AI) was 71%, slightly lower than that obtained for cyclophosphamide (87% at a single i.p. dose of 170 mg/kg). C-1311 administration i.p. at 8 mg/kg/day for 5 consecutive days, gave similar angiogenesis inhibition (AI = 80%).

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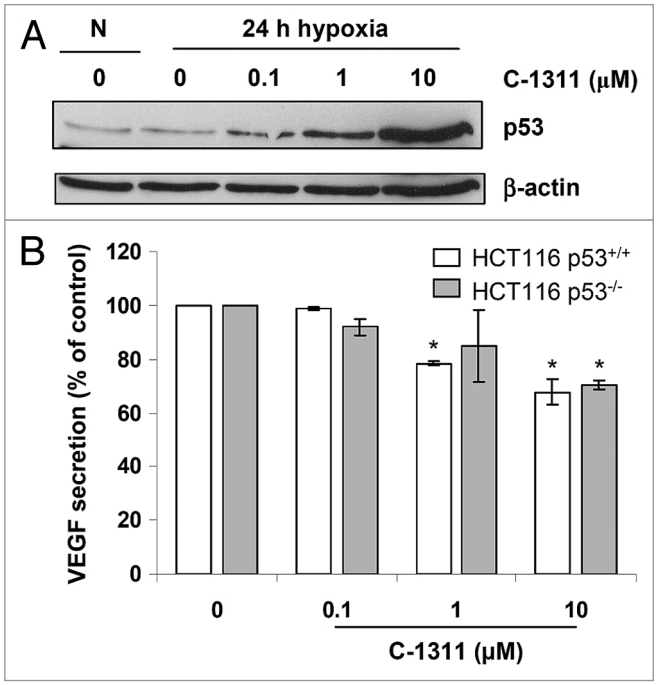

Figure 4. The effect of p53 genotype on hypoxia-induced VEGF protein expression following C-1311 treatment. (A) Protein gel blot analysis of p53 protein level in HCT116 cells. Cells were untreated or exposed to C-1311 for 24 h under hypoxia. Whole-cell lysates (50 μg) were subjected to SDS-PAGE and immunoblotted with antibody against p53. β-actin was used as a loading control. Similar results were observed in three independent experiments. (B) The comparison between the fold changes of VEGF secretion in HCT116 cells with different p53 status. Cells were untreated or exposed to C-1311 for 24 h under hypoxia. The extracellular medium was recovered for VEGF determination by ELISA. Bars represent mean ± range of three independent experiments; *$p < 0.05$ between the indicated concentrations and hypoxia control.

Imidazoacridinone C-1311 inhibits in vivo angiogenesis induced by B16/F10 cells encapsulated in alginate beads. To corroborate the findings from the Matrigel plug assay, we determined the effect of C-1311 on in vivo angiogenesis by using an alginate encapsulation assay.[36] In this approach, s.c. implantation of alginate beads containing tumor cells leads to a strong angiogenic response in recipient mice. To correlate in vivo results with the findings from in vitro studies, we used the B16/F10 mouse melanoma cell line, known for its angiogenic capacity.[36] Alginate implant angiogenesis in female C57BL/6 mice was quantified by measuring the uptake of the blood pool agent FITC-dextran into beads. Quantification of FITC-dextran accumulation within implants, demonstrated that i.p. administration of C-1311 at 8 mg/kg/day for 9 consecutive days led to a complete abrogation of angiogenesis (**Fig. 7B**). In this treatment regimen, alginate implants were colorless and showed an absence of vasculature, whereas implants from untreated mice were reddish and strongly vascularized (**Fig. 7C**). Importantly, as shown in **Figure 7B**, C-1311 even at lower doses of 4 and 6 mg/kg significantly decreased alginate implant angiogenesis by ~40 and ~60%, indicating that inhibitory action of this drug on tumor angiogenesis is dose-dependent.

Discussion

There is compelling evidence warranting the continued efforts to develop new antiangiogenic strategies for anticancer therapies. In this context, new inhibitors intentionally designed to inhibit HIF-1 function are sought.[26] However, anti-HIF-1 properties may also characterize some of the known anticancer agents, especially those that are likely to have multiple cellular targets. In this study, we have shown that a promising DNA-damaging intercalator/alkylator, imidazoacridinone C-1311, inhibits HIF-1α activity in a cell-free system as well as in living cells. C-1311 was also found to inhibit tumor angiogenesis in vivo, uncovering a novel aspect that may be relevant for its anticancer activity.

Our results demonstrate that C-1311 prevents HIF-1α binding to DNA by interacting with HRE sequence. Moreover, C-1311 does not disrupt formation of the protein-DNA complex through direct interference with HIF-1α protein. In a cell-free system, C-1311 inhibited HIF-1α DNA binding activity when the compound was added to the HRE probe directly prior to the addition of hypoxic nuclear extracts, i.e., under conditions which allow for C-1311 intercalation into DNA but disfavor covalent drug binding. Thus, C-1311 intercalation into target sequences may be sufficient to prevent DNA binding of HIF-1α. It has been previously shown that methylation of guanine residues in HREs reduces HIF-1 binding by 100%.[37] Although C-1311 exhibits limited DNA sequence-specificity in its covalent reactivity, following metabolic activation, this compound selectively binds to DNA guanine (Dziegielewski J and Konopa J, manuscript in preparation). Given that covalent binding to DNA is an irreversible process (unlike intercalation), it is interesting to speculate that inhibitory activity of C-1311 on HIF-1α DNA binding may be even more potent in cellular systems which allow for drug covalent binding. Alternatively, while our results provide first evidence that C-1311 interferes with HIF-1α DNA binding, this effect may not be restricted to HIF-1α but may include other transcription factors that have similar core-binding sites or guanine-rich regions in their DNA consensus sequences. One example is the Myc/Max family of transcription factors, whose DNA binding site (E-box, 5'-CAC GTG-3') encompasses the core sequence 5'-CGT G-3', which is also present in the HIF-1 consensus sequence.[38] Although potential inhibition of DNA binding capacity of such transcription factors might detract from the HIF-1α target specificity of C-1311, it may also became a desirable element for its increased therapeutic activity.

Consistent with the observations that C-1311 prevents HIF-1α binding to HRE in a cell-free system, cell-based reporter gene assay showed that C-1311 potently inhibited a HIF-1-dependent gene transcription in hypoxic HCT116 cells. Importantly, C-1311 significantly decreased luciferase activity when pGL2-HRE plasmid harboring the reporter gene driven by a HRE-containing fragment of a VEGF promoter was directly preincubated with C-1311 before cell transfection. Similar results were obtained when cells were treated with C-1311 after pGL2-HRE plasmid transfection. These inhibitory effects confirm that C-1311 downregulates HIF-1 transcriptional activity in vitro by blocking its binding to DNA.

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Whereas C-1311 affects HRE-dependent transcription, the drug does not significantly alter HIF-1α transcription. However, at higher concentrations (10 μM), C-1311 inhibits HIF-1α protein accumulation, as demonstrated for both HCT116 and B16/F10 cells. These observations suggest that in a cellular system, C-1311 may reduce hypoxia-induced HIF-1α activity also by decreasing the amount of HIF-1α protein. One may speculate that the demonstrated decrease in HIF-1α protein level results from proteasomal degradation. It has been recently shown that wild-type tumor suppressor p53 can stimulate the degradation of HIF-1α protein under hypoxia upon treatment with DNA-damaging agents such as mitomycin C,[39] or cisplatin.[40] In our system, C-1311 induced the expression of wtp53 protein in hypoxic HCT116 cells. Thus, wtp53 might be a potential factor to mediate C-1311-induced degradation of HIF-1α under hypoxia. Nonetheless, as shown for the topoisomerase II inhibitor, doxorubicin, degradation of HIF-1α protein can also occur in the absence of p53.[40] Additional studies are clearly required to assess the possibility that C-1311 may stimulate proteasomal degradation of HIF-1α protein.

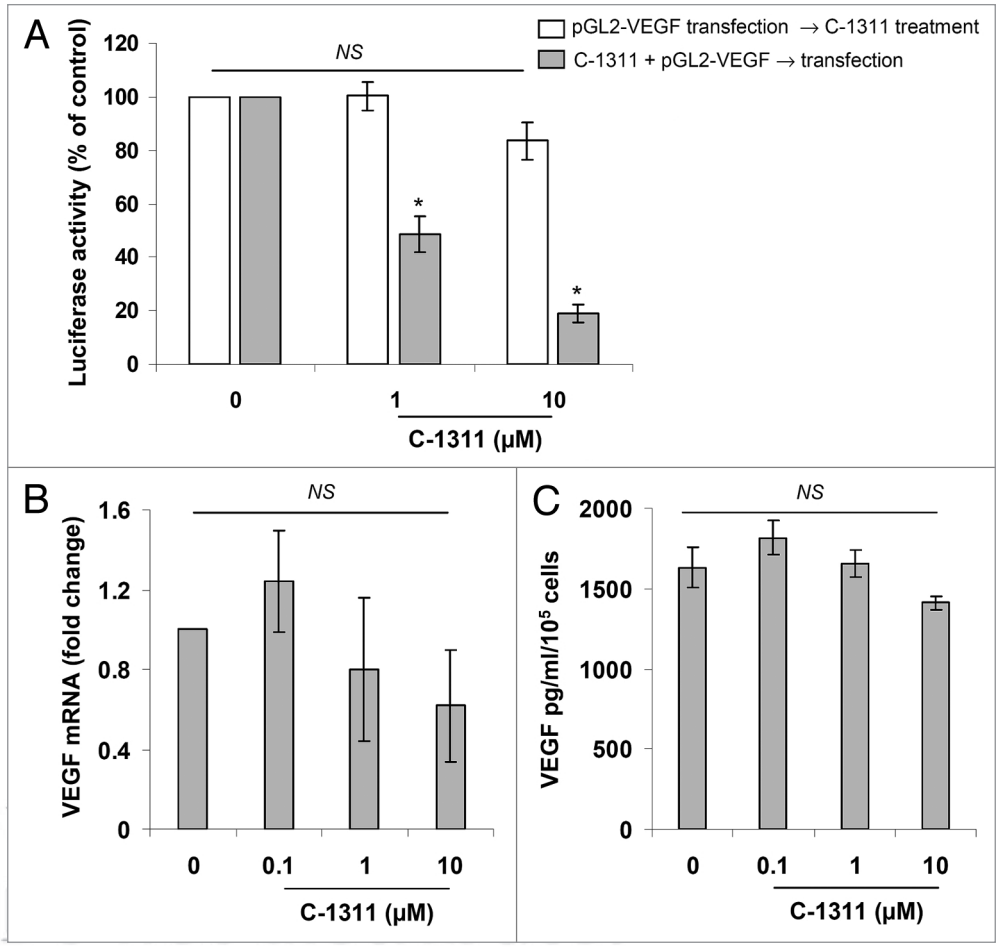

Figure 5. Imidazoacridinone C-1311 does not affect normoxic VEGF mRNA and protein expression in HCT116 cells. (A) HCT116 cells were transiently transfected with a pGL2-VEGF plasmid expressing luciferase under the control of a full-length human VEGF promoter. After transfection, cells were treated with C-1311 under normoxia for 24 h and analyzed for luciferase luminescence assay. Alternatively, pGL2-VEGF reporter plasmid was preincubated with C-1311 for 30 min before cell transfection and cells were subjected to luciferase assay 24 after transfer. Luminescence values were normalized to β-galactosidase expression and presented as percentage of control value (untreated cells under normoxia). Results are the mean ± SE of three independent experiments; *$p < 0.01$ between the indicated concentrations and normoxia.
(B) Cells were untreated or exposed to C-1311 under normoxia for 48 h. VEGF mRNA levels were assessed by real-time PCR and normalized to β-actin mRNA levels. Results are expressed as fold change relative to VEGF mRNA levels under normoxic conditions (equal to 1) in the absence of drug. The bars represent the mean ± range of three independent experiments. (C) HCT116 cells were incubated in normoxic conditions in the presence of C-1311 for 48 h. The extracellular medium was recovered for VEGF determination by ELISA. VEGF level expressed as picogram per milliliter per 10^5 cells. Bars represent mean ± range of three independent experiments.

In general, the inhibition of HIF-1α DNA binding capacity as well as the decrease in HIF-1α protein levels is associated with a decrease in HIF-1α transactivating activity leading to the decreased expression of one of the major downstream targets of HIF-1, VEGF.[31] Consistent with this scenario, in HCT116 cells C-1311 very effectively inhibited activity of a full-length VEGF promoter under hypoxia, as demonstrated by reporter gene assay. C-1311 also reduced hypoxia-induced VEGF mRNA expression in a time- and dose-dependent manner. The functional relevance of these findings is underscored by the demonstration that VEGF mRNA inhibition was associated with suppression of VEGF protein secretion. Importantly, in HCT116 and B16/F10 cells, upon prolonged exposure, C-1311 significantly reduced hypoxia-induced VEGF expression, whereas inhibition of HIF-1α expression was much more modest, suggesting that downregulation of HIF-1α at the protein level plays a secondary role in C-1311-induced VEGF inhibition through the HIF-1α/HRE system.

Interestingly, C-1311 decreased hypoxia-induced VEGF protein production irrespective of p53 tumor suppressor protein status (wt vs. lack of p53). This finding is of particular interest given that the inactivation of p53, which occurs in approximately half of tumors, augments HIF-1-dependent expression of VEGF; in consequence tumors without p53 or carrying p53 mutations appear more vascularized and are often more aggressive and correlate with poor prognosis.[41] It is tempting to speculate that due to the demonstrated ability of C-1311 to decrease VEGF expression

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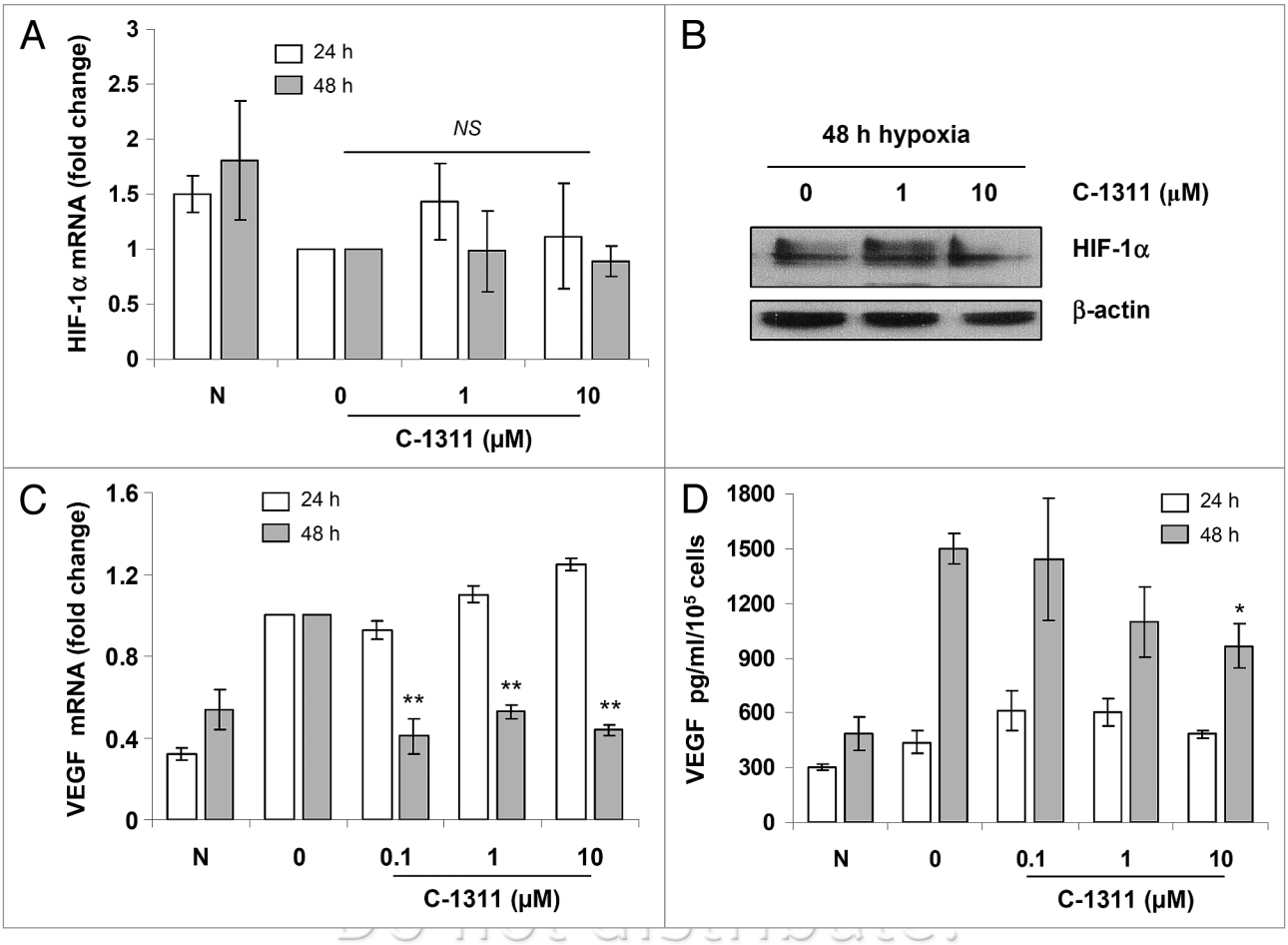

Figure 6. The effect of imidazoacridinone C-1311 on HIF-1α and VEGF expression in murine melanoma B16/F10 cells. Cells were incubated in hypoxic conditions in the presence of C-1311 for 24 and 48 h. HIF-1α (A) and VEGF (C) mRNA levels were assessed by real-time PCR and normalized to β-actin mRNA levels. Mean relative expression ratios of HIF-1α/β-actin or VEGF/β-actin mRNA are shown. Non-treated B16/F10 cells under hypoxia were set at 1. The bars represent the mean ± range of three independent experiments; $^{**}p < 0.01$ between the indicated concentrations and hypoxia control. (B) Protein gel blot analysis of HIF-1α protein following 48 h of C-1311 treatment under hypoxia. Nuclear extracts 50 (μg) from B16/F10 cells were subjected to SDS-PAGE and immunoblotted with antibody against HIF-1α. β-actin was used as a loading control. (D) B16/F10 cells were incubated in hypoxic conditions in the presence of C-1311 for 24 and 48 h. The extracellular medium was recovered for VEGF determination by ELISA. VEGF level expressed as picogram per milliliter per 10^5 cells. Bars represent mean ± range of three independent experiments. $^{*}p < 0.05$ between the indicated concentrations and hypoxia control. *N*, untreated cells under normoxia.

in p53-deficient cancers, such cancers may be particularly good candidates for C-1311 treatment.

It is well known that VEGF expression is regulated by HIF-1-dependent and HIF-1-independent mechanisms.[31] Our studies revealed that in normoxia, when HIF-1 is not the major factor regulating VEGF transcription, C-1311 failed to suppress VEGF expression. In normoxic HCT116 cells, C-1311 did not significantly affect either VEGF mRNA level or VEGF protein secretion. Moreover, although C-1311 inhibited activity of a full-length VEGF promoter when pGL2-VEGF plasmid was preincubated with C-1311 before cell transfection, the drug did not suppress VEGF promoter activity when HCT116 cells were treated with C-1311 following plasmid transfection. It seems that in cellular system, under normoxic conditions, binding of C-1311 within VEGF promoter does not affect its functionality. Given that in case of normoxia, VEGF expression is mediated mainly by the SP-1 protein,[42] while the most important transcription factor responsible for hypoxia-induced generation of VEGF is HIF-1, these results further emphasize the importance of the HIF-1 pathway in C-1311 inhibition of VEGF expression.

The demonstrated ability to negatively influence HIF-1α activity with a concomitant reduction of VEGF expression suggests that C-1311 may have antiangiogenic properties in addition to its other known activities. This possibility is reinforced by the direct demonstration of C-1311 inhibitory activity in two in vivo angiogenesis assays, i.e., Matrigel and alginate encapsulation assays. Our results show for the first time that C-1311 inhibits neovascularization of Matrigel plugs and alginate implants in mice in a dose-dependent manner. In general, there are two classes of angiogenesis inhibitors: "direct" and "indirect." Direct angiogenesis inhibitors prevent vascular endothelial cells from proliferating, migrating, or responding to angiogenic proteins, including VEGF, bFGF and others. Indirect angiogenesis inhibitors prevent the expression or block the activity of the proteins

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that activate angiogenesis.[43] The ability of C-1311 to reduce both VEGF expression in B16/F10 cells in vitro and angiogenesis triggered by the same tumor B16/F10 cells encapsulated in alginate beads in vivo not only suggest the causative link between these effects, but also indicate that C-1311 is an indirect angiogenesis inhibitor. It is reasonable to assume that the inhibitory action of C-1311 on the tumor cells itself results in an inability to release angiogenic growth factors. On the other hand, the finding that C-1311 significantly blocked angiogenesis stimulated by bFGF alone locally released from the Matrigel sponge, points to the direct effect of C-1311 on endothelial cells. It is well established that C-1311 exhibits high anti-proliferative and cytotoxic properties when tested on tumor cell lines.[10-15] Although it needs further investigation, it is reasonable to hypothesize that this drug may also exert its inhibitory effect on endothelial cell growth and survival, thus preventing their recruitment into hypoxic and vascular areas. Nonetheless, given that Matrigel and alginate encapsulation assays, both widely used in vivo angiogenesis assays, reflect many of the features of tumor angiogenesis, it is plausible to expect that this drug may also show antiangiogenic properties in future patients.

Conventional chemotherapy, which is administered at more toxic "maximum tolerated doses," requires 2–3 wk breaks between successive cycles of therapy. Instead of only using short bursts of toxic MTD chemotherapy, there is now a shift in thinking toward the anti-angiogenic effects of chemotherapy which seem to be optimized by administering chemotherapeutics 'metronomically'—in small doses on a frequent schedule (even daily) in an uninterrupted manner, for prolonged periods.[44] In this study, we found that even a single i.p. administration of C-1311 of 40 mg/kg to mice resulted in significant inhibition of Matrigel angiogenesis, whereas the approximate MTD of C-1311 given to mice was 200 mg/kg as a single i.p. dose (data not shown). Importantly, treatment of mice with C-1311 on a different schedule e.g., 8 mg/kg/day i.p. for 5 d resulted in similar degree of angiogenesis inhibition. These results suggest that schedules of frequent C-1311 administration using much smaller individual doses may be more effective in terms of reducing the level of toxicity, therefore making more prolonged C-1311 treatment possible.

In summary, this study provides the first evidence that anti-proliferative and antitumor effects of C-1311 may involve interference with HIF-1α binding to HRE, resulting in decreased hypoxia-induced VEGF expression. Importantly, we show that C-1311-driven VEGF inhibition may result in the reduction of in vivo angiogenesis. Further studies are needed to establish the detailed molecular basis and the significance of the antiangiogenic activity of C-1311 for its overall antitumor properties. C-1311 is clearly a multitargeted compound already recognized as: (a) a DNA-damaging agent; (b) topoisomerase II inhibitor/poison; (c) FLT3 tyrosine kinase inhibitor; (d) quinone oxidoreductase NQO2 inhibitor; and (e) as revealed herein

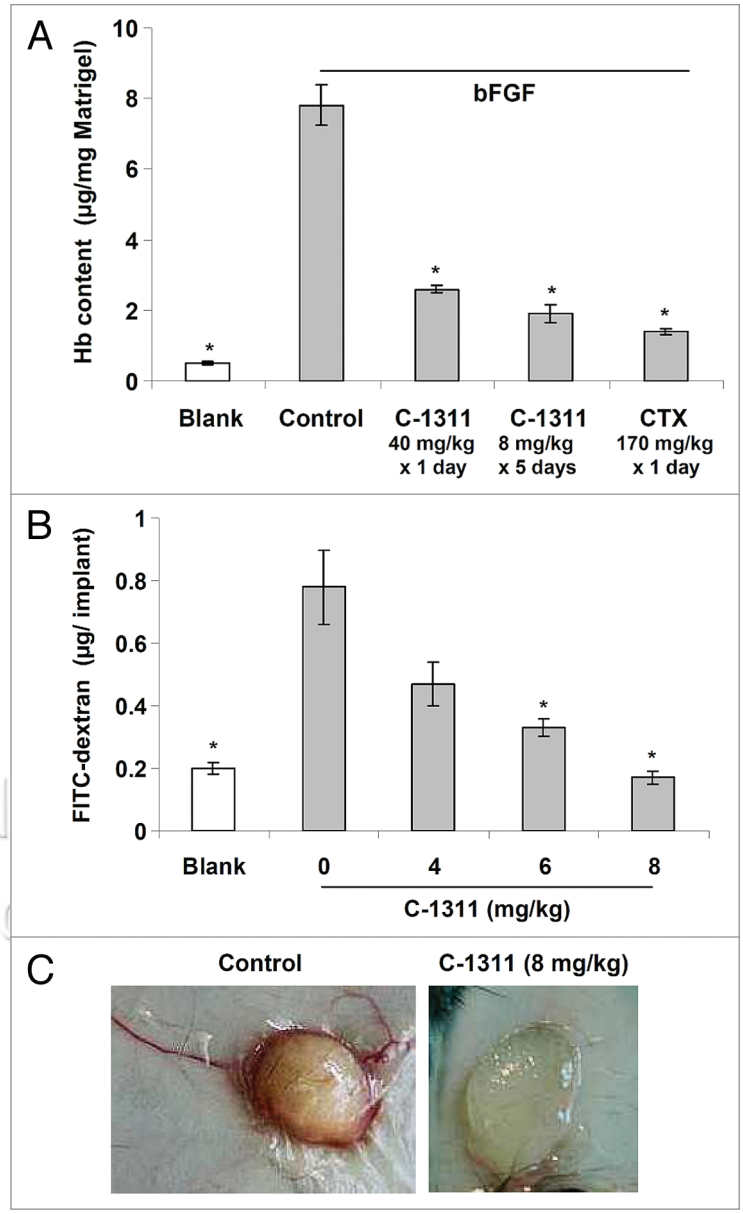

Figure 7. Imidazoacridinone C-1311 strongly inhibits angiogenesis in vivo. (A) C57BL/6 mice were implanted s.c. with Matrigel sponges that contained no bFGF (*Blank*) or bFGF alone, as described in the Materials and Methods. After 1 d of Matrigel plug implantation, mice received either vehicle alone (*Control*), or C-1311 i.p. as a single dose of 40 mg/kg, or as five daily doses of 8 mg/kg. A single dose of cyclophosphamide (170 mg/kg, *CTX*) served as a positive control. Seven days later, Matrigel sponges were surgically removed and hemoglobin (*Hb*) content in the plugs was quantified as an indicator of blood vessel formation. Results are expressed as mean ± range from three independent experiments. In a single experiment, at least eight animals were used in each treatment group. *$p < 0.01$ compared with vehicle control. (B) C57BL/6 mice were implanted s.c. with alginate beads that contained no B16/F10 cells (*Blank*) or 1 x 10^5 B16/F10 cells, as described in the Materials and Methods. After 1 d of beads implantation, mice received either vehicle alone or C-1311 i.p. as nine daily doses. Nine days later, beads were surgically removed and the blood pool agent FITC-dextran accumulation in alginate implants was measured as an indicator of blood vessel formation. Results are expressed as mean ± range from three independent experiments. In a single experiment, at least eight animals were used in each different treatment group. *$p < 0.01$ compared with vehicle control. (C) Representative photographs of alginate implants show a significant decrease in vascularization following C-1311 treatment.

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HIF-1α, VEGF and angiogenesis targeting agent. The clinical success of multitargeted compounds indicates that chances of success in eradicating cancer will increase when catching tumors in the crossfire of more than one pathway-inhibiting agent.[45,46] In this context, the identification of other preferential targets of C-1311 warrants further efforts toward its clinical development as an anticancer drug.

Materials and Methods

Chemicals and reagents. Imidazoacridinone C-1311 was synthesized at the Gdansk University of Technology (Poland), as described previously in reference 1, and provided by Dr. B. Horowska from our Department. Cyclophosphamide was purchased from ASTRA Medica Solutions of the drugs were freshly prepared before administration in saline. Matrigel was purchased from BD Bioscience, and basic fibroblast growth factor (bFGF) was from CALBIOCHEM. All other reagents, unless stated otherwise, were from Sigma-Aldrich.

Cell lines and animals. Human colon adenocarcinoma HCT116 cell line and its p53-deficient variant (HCT116 $p53^{-/-}$) were kindly provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD) and have been described previously in reference 47. Cells were grown in Mc'Coys medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO Life Technologies) at 37°C in a humidified incubator containing 21% O_2, 5% CO_2 in air (referred to as normoxic conditions). The B16/F10 mouse melanoma cell line was obtained from Dr. J. Hoffman (Max-Delbrück Center for Medicine, Berlin, Germany) and has been described previously in reference 36. Cells were cultured under normoxic conditions in MEM supplemented with 5% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. For treatment under hypoxic conditions, HCT116 and B16/F10 cells were placed in a hypoxic chamber, thoroughly flushed for 20 min with a mixture of 1% O_2, 5% CO_2 and 94% N_2, tightly sealed, and then incubated at 37°C. Alternatively, hypoxia mimetic agent, cobalt chloride ($CoCl_2$), was used at final concentration of 300 μM. The C57BL/6 mice were purchased from the Inbred Mouse Breeding Center, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Warsaw, Poland. Mice received water and food ad libitum. In vivo studies were performed in accordance with established institutional guidance and approved protocols.

Nuclear cell extracts preparation and ELISA for HIF-1α DNA binding. HCT116 cells were seeded in 100-mm Petri dishes at 2.5 x 10^6 cells/dish and incubated overnight. The next day, the medium was replaced with fresh medium and cells were subjected to hypoxia for 16 h to induce HIF-1α expression. Nuclear extracts were prepared using the Nuclear Extract kit (Active Motif) and kept at -80°C before use. Hif-1α DNA-binding activity was determined using the ELISA TransAM kit (Active Motif). Briefly, different concentrations of imidazoacridinone C-1311 were added directly to individual wells of a 96-well plate coated with oligonucleotide containing the consensus-binding sequence of HIF-1α 5'-TAC GTG CT-3' (HRE probe). After 15 min, 6-μg aliquots of nuclear extracts were added to the plate and incubated at room temperature for 1 h. Alternatively, hypoxic nuclear extracts were directly incubated with different concentrations of C-1311 for 15 or 40 min at room temperature. After preincubation, C-1311 and nuclear extract mix was added to the plate coated with DNA HRE probe. The appropriate primary and horseradish peroxidase-labeled secondary antibodies, included in the kit, were used to detect bound HIF-1α. HIF-1α DNA binding ELISA results were read in a plate reader by measuring absorbance at 450 nm with correction at 540 nm. Nuclear extracts from normoxic HCT116 cells and hypoxic nuclear extracts incubated with a HIF-1α-specific competitor oligonucleotide (Actif Motif) served as negative and positive controls.

Reporter gene assay and luminescence measurements. To assess the effect of C-1311 on HIF-1 transcriptional activity or VEGF promoter, HCT116 cells were transfected with a reporter plasmid pGL2, containing the luciferase cDNA regulated by a HRE fragment of VEGF promoter (-1,014 to -903) or by the human full-length VEGF promoter (+54 to -2,279). Plasmids were constructed by Dr. Hideo Kimura (Japan) [29] and kindly provided by Prof. Józef Dulak (Poland). HCT116 cells grown to 70% confluence were transfected in 24-well plates using 0.5 μg DNA per well and FuGene HD Transfection Reagent (Roche). After transfection, cells were exposed to C-1311 under hypoxia for 24 h. Alternatively, reporter plasmids were preincubated with C-1311 for 30 min before cell transfection and cells were subjected to luciferase assay 24 after transfer. To control transfection efficiency, cells were cotransfected with 0.1 μg pSV-β-galactosidase vector (generous gift from Prof. Dulak). Luciferase activity was measured using the Luciferase Assay System (Promega). β-galactosidase activity was measured using β-galactosidase Enzyme Assay System (Promega). The luciferase activity of the transfectants was compared after normalizing their β-galactosidase activity.

Protein gel blot analysis. Cells were grown to 80% confluence in 100-mm Petri dishes and treated with different concentrations of C-1311 in hypoxia or with hypoxia-mimicking cobalt chloride. Cells were collected and nuclear extracts were prepared using the Nuclear Extract kit (Active Motif). Proteins (50 μg) from nuclear extracts were run on 10% SDS-PAGE, transferred to nitrocellulose membrane, and stained with monoclonal mouse anti-human HIF-1α antibody (1:200; BD Transduction Laboratories), monoclonal mouse anti-mouse HIF-1α antibody (1:200; R&D Systems), and monoclonal anti-β-actin antibody (1:5,000; Sigma-Aldrich). To determine the p53 protein level, cells were lysed in a buffer containing 50 mM Tris-base pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P40 and 1 mM PMSF supplemented with protease inhibitors cocktail (Roche Diagnostics). Proteins (50 μg) from whole-cell lysates were run on 12% SDS-PAGE, transferred to nitrocellulose membrane, and stained with monoclonal anti-p53 antibody (1:500; Sigma-Aldrich) and monoclonal anti-β-actin (1:5,000; Sigma-Aldrich). For each protein gel blot, chemiluminescence detection was performed using ECL reagents (Thermo Scientific).

Real-time PCR. Total cellular RNA was obtained with the High Pure RNA Isolation kit (Roche Diagnostics). cDNA

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was synthesized by reverse transcribing total RNA (1 μg) with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics). Gene expression of HIF-1α, VEGF and β-actin were detected by real-time PCR. The primer sequences for human and mouse genes were those described by Fukuda et al. and Kim et al. Real-time PCRs were performed in a LightCycler 1.8 using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics). The thermal cycling conditions were as follows: initial incubation of 3 min at 95°C, followed by 45 cycles of 30 sec at 95°C, 1 min 59°C and 30 sec at 72°C. All cDNA samples were tested in duplicate and at the end of the PCR cycles, a melting curve was generated to determine specificity of the PCR product. Samples were compared using the relative Ct (threshold Cycle) method. Relative quantitation values were expressed as follows: $2^{(\Delta Ctr - \Delta Ctt)}$, where Ct is the calculated cycle number at which the fluorescence signal is significantly above background levels, ΔCt is the difference between the mean Ct values of the target gene and those of the endogenous control for the same sample, ΔCtr - ΔCtt represents the difference between the mean ΔCt of the reference (non-treated) sample and ΔCt of the drug-treated samples under hypoxia. Values are expressed as fold increase relative to the non-treated sample under hypoxia. β-actin mRNA was used as an endogenous control.

Determination of VEGF levels in culture medium. Cells were seeded in a 24-well plate at 5 x 10^4 cells/well and incubated overnight in medium containing 10% FBS. The medium was then replaced with medium containing 1% FBS. Cells were subjected to normoxic or hypoxic conditions for 24 or 48 h in the presence of various doses of C-1311. VEGF concentrations in the conditioned media were quantified by Human Quantikine VEGF Immunoassay kit (R&D Systems) and Mouse VEGF-A Platinum ELISA (eBioscience). Secreted VEGF was determined using an ELISA measuring $VEGF_{165}$ and $VEGF_{121}$ isoforms. VEGF concentrations were quantified by comparison with the series of VEGF standard samples included in the assay kit. The results were expressed as concentrations of VEGF (picogram per milliliter) per number of cells in each well.

Mouse matrigel plug assay. The Matrigel plug assay was performed as previously reported in reference 34. Briefly, female C57BL/6 mice were injected s.c. in the ventral site with 0.5 mL of chilled Matrigel containing 60 U/mL heparin, with or without 160 ng/mL bFGF in 0.1% BSA in PBS. Starting from day 1 after Matrigel implantation, C57BL/6 females received a single dose of C-1311 (40 mg/kg) or were treated daily with C-1311 at a dose of 8 mg/kg for 5 consecutive days. Animals injected with a single 170 mg/kg dose of cyclophosphamide served as a positive control. After 7 d, the Matrigel plugs were recovered, minced and dispersed in water. Hemoglobin content within the plugs was measured as an indicator of angiogenesis using the Drabkin reagent kit (Sigma-Aldrich). Results were normalized to the weight of the Matrigel plugs and reported as μg of hemoglobin/mg of plug. The degree of angiogenesis inhibition (AI) was calculated using the following formula: AI = 100 - 100 x $[Hg_t - Hg_{blank}]/[Hg_c - Hg_{blank}]$ (%), where Hg_t is the concentration of hemoglobin in Matrigel plugs with bFGF in treated mice, Hg_c is the concentration of hemoglobin in plugs with bFGF in control mice, and Hg_{blank} is the concentration of hemoglobin in plugs without bFGF in untreated mice.

Alginate-encapsulated tumor cell assay. Tumor angiogenesis inhibition studies were performed using a model for quantification of in vivo angiogenesis induced by alginate-encapsulated tumor cells, as described by Hoffmann et al. Briefly, proangiogenic B16/F10 mouse melanoma cells, harvested from cell culture, were resuspended with the 1.5% sodium alginate solution in sterile saline to the desired cell number (1 x 10^5/mL). Droplets containing tumor cells were produced by extrusion of the alginate solution trough an 8-gauge cannula directly into a swirling bath of 80 mM $CaCl_2$. The size of the beads was minimized by a laminar flow along the cannula. After incubation in the $CaCl_2$ bath for an additional 30 min, the beads were washed three times with MEM medium without FBS, centrifuged for 10 min at 600x g, and prepared for injection. Next, female C57BL/6 mice were injected s.c. with 0.1 mL of alginate beads into the upper third of the back. Control mice were implanted with 0.1 mL of alginate beads without tumor cells. After 24 h for bead implantation, C-1311 was administered i.p. once a day for 9 consecutive days. At the end of the experiment, 0.2 mL of 1% FITC-dextran solution (100 mg/kg body weight) was injected into the lateral tail vein. Twenty min after FITC-dextran injection, alginate implants were rapidly removed and transferred to tubes containing 2 mL saline. Samples were homogenized in the dark at room temperature and centrifuged for 3 min at 1,000x g. Supernatant fluorescence was measured by excitation at 492 nm and emission at 515 nm. The FITC-dextran accumulation was quantified by comparison with calibration curves (0.01–10 μg/mL FITC-dextran in saline).

Statistical analysis. Unless indicated otherwise, the results are presented as mean ± SE of three independent experiments. Experimental data were analyzed using a one-way ANOVA. p values below 0.05 were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

Acknowledgments

This work was supported by Polish State Committee for Scientific Research (K.B.N.) grant 3P05A 117 24 and partially by National Center for Research and Development (NCBIR) grant 13-0133-10/2011. The authors thank Professor Józef Dulak and Dr. Agnieszka Łoboda for their valuable assistance with the gene reporter assay. The authors also thank Dr. Jan M. Woynarowski for critical reading of the manuscript, insightful comments and valuable suggestions.

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