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A novel benzimidazole analogue inhibits the hypoxia-inducible factor (HIF)-1 pathway

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ABSTRACT

Hypoxia-inducible factor (HIF)-1 is a therapeutic target in solid tumors. We report the novel benzimidazole analogue AC1-004, obtained from a chemical library using an HRE-dependent cell-based assay in colorectal carcinoma HCT-116 cells. The accumulation of hypoxia-induced HIF-1α was inhibited by compound AC1-004 in various cancer cells, including HCT-116, MDA-MB435, SK-HEP1, and Caki-1. Further, AC1-004 down-regulated VEGF and EPO, target genes of HIF-1, and inhibited in vitro tube formation of HUVEC, suggesting its potential inhibitory activity on angiogenesis. Importantly, AC1-004 was found to regulate the stability of HIF-1α through the Hsp90-Akt pathway, leading to the degradation of HIF-1α. An in vivo antitumor study demonstrated that AC1-004 reduced tumor size significantly (i.e., by 58.6%), without severe side effects. These results suggest the benzimidazole analogue AC1-004 is a novel HIF inhibitor that targets HIF-1α via the Hsp90-Akt pathway, and that it can be used as a new lead in developing anticancer drugs.

Hypoxia-inducible factor (HIF)-1 is a heterodimeric basic-helix–loop–helix–PAS domain transcription factor consisting of HIF-1α and HIF-1β [1,2]. Of these, HIF-1α is regulated by O_2-dependent prolyl hydroxylation in the ODD region [3], which is required for ubiquitinylation by E3 ubiquitin–protein ligases—including von Hippel–Lindau tumor-suppressor protein (VHL)—and subsequent proteasomal degradation [4,5]. The HIF-1α expression level increases in response to hypoxia in tissues, and functional HIF-1α up-regulates target genes by binding to the hypoxia-responsive elements (HREs) of regulatory regions.

HIF-1 is considered a central regulator of the adaptation responses of cancer cells to hypoxia [6–8] and is responsible for gene expressions that influence angiogenesis, modulation glucose metabolism, cell proliferation, survival, and invasion in solid tumors during tumor progression and metastasis [9–14]. Intratumoral hypoxia and genetic alterations can lead to HIF-1α overexpression [15]. In animal models, HIF-1α overexpression is associated with increased tumor growth, vascularization, and metastasis, whereas HIF-1 loss-of-function has the opposite effect; these findings validate HIF-1α as an attractive target [15–17]. Recently, considerable effort has been directed to the discovery of HIF-1 inhibitors, from chemical libraries and natural products alike [18–26]. These inhibitors reportedly regulate the HIF-1 signaling pathway through a variety of molecular mechanisms, including transcriptional regulation, folding, stabilization, nuclear translocation, degradation, and transactivation.

Our project currently focuses on the development of small-molecule inhibitors that target HIF-1α protein in hypoxic condition [27,28]. To identify a new HIF-1α inhibitor, a chemical library constructed via the derivatization of (aryloxyacetylamino) benzoic acid analogue **1** was screened using a cell-based HRE-dependent reporter assay (Fig. 1A). Previously, analogues **2** and **3** were evaluated as HIF-1α inhibitors [27,28]. Herein, we report our recent discovery of a benzimidazole analogue **4** (AC1-004), which was found to regulate the stability of HIF-1α through the Hsp90-Akt pathway and inhibit tumor growth by 58.6% in a mouse model.

Materials and methods

Materials. Chemicals, media, cell culture reagents, and materials—including LY294002 and MG132—were purchased from Life

Abbreviations: HIF-1, hypoxia-inducible factor 1; HRE, hypoxia responsive element; VEGF, vascular endothelial growth factor; EPO, erythropoietin; HUVEC, human umbilical vascular endothelial cell

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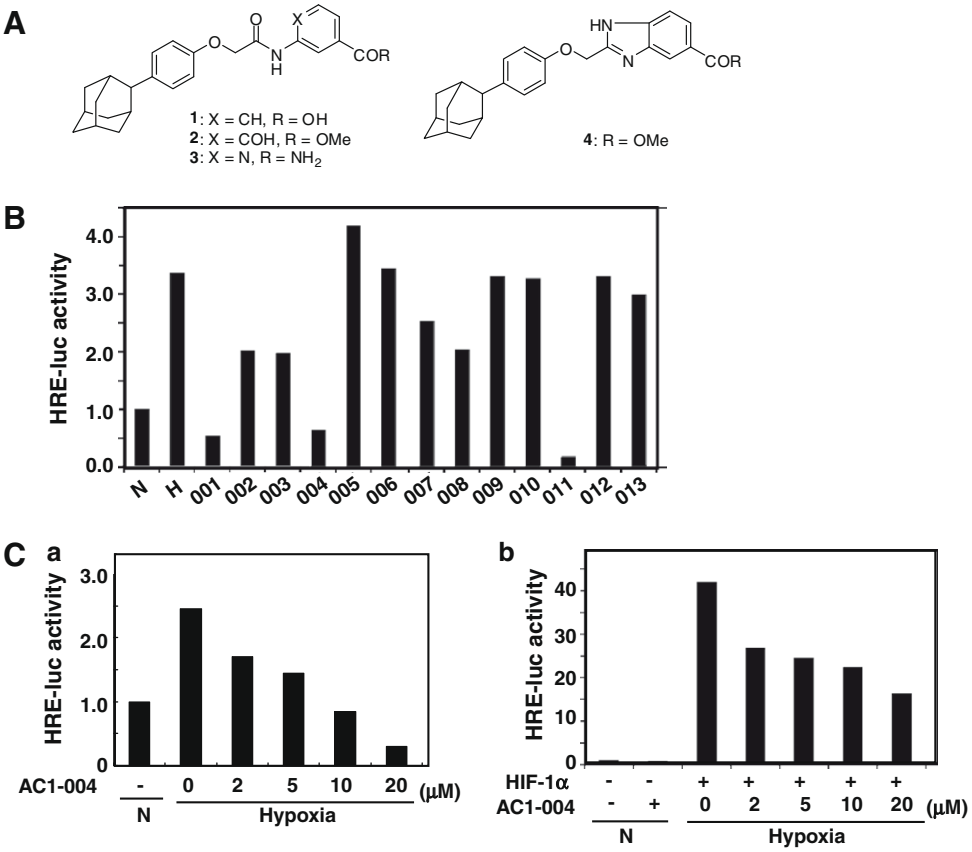

Fig. 1. Identification of a novel benzimidazole analogue by HRE-dependent luciferase reporter assays. HCT-116 cells were transiently co-transfected with pGL3-HRE-Luc plasmid containing six copies of HREs from human VEGF gene and pRL-SV40 plasmid-encoding Renilla luciferase. The luciferase assay was carried out using a dual-luciferase reporter assay system. After 12 h of incubation, the cells were treated with various concentrations of the tested compounds and incubated for 16 h in hypoxic condition. (A) Structure of small molecule HIF-1α inhibitors. (B) Screening of chemical library using a HRE-luciferase assay. A total of 300 compounds were screened; the figure shows 13 compounds in the second screening. "00*n*" stands for AC1-00*n*. (C) a. Inhibition of HRE-dependent luciferase activity by AC1-004. Different concentrations were used to determine IC_{50} of AC1-004. b. Inhibition of HRE-dependent luciferase activity by AC1-004, in cells transiently expressing HIF-1α. HRE-luciferase assay by AC1-004 was performed in the cells co-transfected with pGL3-HRE-Luciferase and the plasmid containing HIF-1α.

Technologies, Inc. (Gaithersburg, MD, USA), Sigma (St. Louis, MO, USA), Fisher Scientific (Fairlawn, NJ, USA), and Corning, Inc. (Corning, NY, USA). Anti-β-actin, anti-HIF1α, and anti-HIF1β antibodies were purchased from BD Biosciences (Frankline Lakes, NJ, USA). Anti-phospho-Akt, anti-Akt, anti-phospho-GSKβ, and anti-GSKβ antibodies were purchased from Cell Signaling (Danvers, MA, USA), and anti-CDK4 and anti-Cyclin D1 antibodies from Santa Cruz (Santa Cruz, CA, USA). Matrigel™ and reagents for in vitro tube formation assay were also purchased from BD Biosciences.

Chemical synthesis. The synthesis and characterization of AC-004 are described in the Supplementary material.

Cell culture. Human colorectal carcinoma HCT-116 cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS; Lonza, Inc.). Caki-1 (human Caucasian kidney carcinoma), SK-HEP1 (hepatocellular carcinoma), and MDA-MB435 (originally identified as a breast cancer cell line, but reclassified as a melanoma cell line; http://dtp.nci.nih.gov/docs/misc/common_files/mda-mb-435-update.html) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Lonza, Inc.). All media contained 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). All cells were cultured in an atmosphere of 5% CO_2 at 37 °C, and hypoxia was induced by culturing cells in a hypoxia chamber flushed with a mixed gas of 1% O_2, 5% CO_2, and 94% N_2.

Reporter assay. The ability of the compounds to inhibit HIF-1 was determined by a reporter assay, as previously described [27]. At 75–90% confluence, HCT-116 cells were transiently co-transfected with pGL3-HRE-Luciferase plasmid containing six copies of HREs from human VEGF genes and pRL-SV40 plasmid-encoding renilla luciferase (Promega, Madison, WI, USA). After 12 h of incubation, the cells were treated with various concentrations of the tested compounds and incubated for 16 h in hypoxic condition. The luciferase assay was performed using a dual-luciferase reporter assay system (Promega). Luciferase activity was determined in a Microlumat Plus luminometer (EG&G Berthold, Bad Wildbad, Germany). The results were normalized to the activity of renilla luciferase expressed by the co-transfected Rluc gene, under the control of a constitutive promoter.

Western blot analysis. Cells were lysed by adding sodium dodecyl sulfate (SDS) sample buffer and 0.03% (wt/vol) bromophenol blue. Total cell lysates were denaturated by boiling for 5 min, resolved on SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffer saline containing 5% (wt/vol) skim milk and 0.1% Tween 20 for 2 h; they were then incubated with a primary antibody overnight, at 4 °C. The blot was developed using a horseradish peroxidase-conjugated secondary antibody (phototope-horseradish peroxidase Western blot detection kit; Millipore).

RNA extraction and RT-PCR. Total RNA was extracted from cells by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA samples (1 μg) were subjected to reverse transcription using the Maxime RT PreMix for cDNA synthesis, which was used as a template PCR premix (Bioneer). The primer sequences used were as follows:

VEGF forward, 5′-GGTGGACATCTTCCAGAGTA-3′; VEGF reverse, 5′-GGCTTGTCACATCTGCAAGTA-3′; EPO forward, 5′-TATGCCT GGAA-GATGGAGGTC-3′; EPO reverse, 5′-TGTCAGCAGTGATTGTTCG-GAAG-3′; HIF1α forward, 5′-CTATATCCCAATGGATGATGATGA-3′; HIF1α reverse, 5′-ATCATGTTCCATTTTTCGCTT-3′; GAPDH forward, 5′-ATGGGGAAGGTGAAGGTCGG-3′; and GAPDH reverse, 5′-CAG-GAGGCATTGCTGATGAT-3′.

In vitro tube formation assay. The wells of a 96-well plate were coated with ice-cold BD Matrigel™ matrix gel solution. After polymerizing the matrix at 37 °C, human umbilical vein endothelial cells (HUVECs) were seeded onto the polymerized EC matrix at a concentration of 1×10^4 cells in 180 μl of EMB-2 media per well; 20 μl of the sample was immediately added. The tubule branches were photographed after 16 h of incubation. The results of three independent experiments are given.

In vivo animal model. The in vivo antitumor activity of AC1-004 was evaluated in mice using MDA-MB-435 cells (four to six-week-old female athymic nude mice, Crj:BALB/c nu/nu; Charles River). When the tumor volume reached approximately 100 mm^3, the mice received the following treatment every other day, via intraperitoneal (i.p.) injection: group 1 (control group; six mice), vehicle solution; group 2 (six mice), AC1-004 at a dose of 20 mg/kg per animal; and group 3 (six mice), AC1-004 at a dose of 50 mg/kg per animal. The treatments were continued for 5 weeks. Tumor volume (V) was determined using the following equation:

$$V = (L \times W^2) \times 0.5,$$

where L, long side and W, short side. Tumor growth inhibition was analyzed for statistical significance, using a Student's t-test [29].

Results and discussion

Identification of novel benzimidazole analogue by HRE-dependent luciferase reporter assays

The active HIF-1 recognizes and binds to the HREs (5′-A/GCGTG-3′) present in hypoxia-inducible promoters [30,31]. A total of 300 compounds from our in-house synthetic chemical library were screened for their potential to inhibit hypoxia-induced HIF-1α activity via a luciferase reporter gene, which is under the control of HRE of VEGF promoter [27]. The chemical library was constructed via derivatization of (aryloxyacetylamino) benzoic acid analogue 1 (AC-001) (Fig. 1A). As shown in Fig. 1B, compounds selected from the initial screening were re-evaluated in a second screening, at a concentration of 30 μM. The luciferase activity in the HCT-116 cells increased under hypoxia via accumulation of HIF-1α protein. Upon treatment with the benzimidazole analogue AC1-004 (4), the inhibitory activity was observed similar to that of AC1-001 [27]. AC1-004 was chosen for further study, due to its novel structure and potency.

It was found that AC1-004 inhibited HRE dependent reporter activity in a dose-dependent manner under hypoxia, exhibiting IC_{50} value of 1.8 μM (Fig. 1Ca). To address whether or not the suppression of the HRE promoter by AC1-004 was mediated by the inhibition of HIF-1α, the inhibitory activity was assayed in the cells transiently expressing HIF-1α as shown in Fig. 1Cb. Luciferase activity increased in 40-fold in the cells expressing HIF-1α under hypoxia. However, upon treatment with AC1-004, HRE-Luc activity was decreased gradually, with the increase in concentration suggesting that AC1-004 inhibited HRE activity via HIF-1α protein.

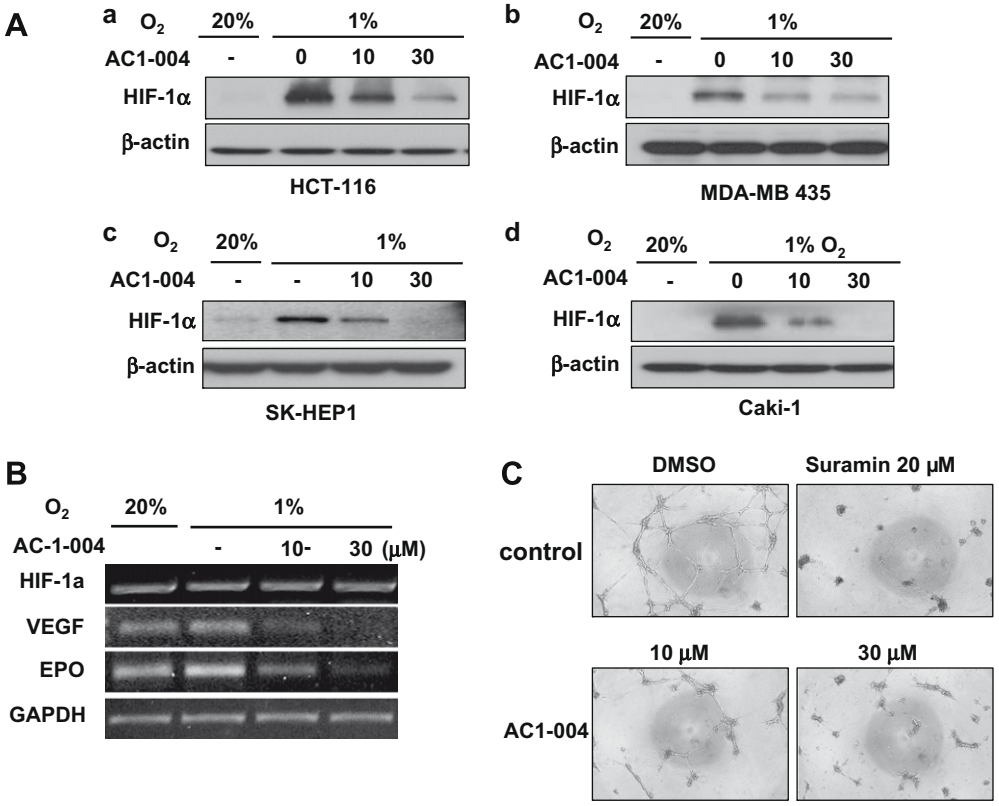

Fig. 2. The effect of the benzimidazole analogue AC1-004 on HIF-1α. (A) Effects on hypoxia-induced HIF-1α accumulation. The effect of AC1-004 on HIF-1α expressions in various human carcinoma cancer cell lines (SK-HEP1, MDA-MB-435, HCT-116, and Caki-1) was determined by Western blot analysis. (B) Effect of AC1-004 on the mRNA levels of HIF-1 target genes. The mRNA levels of target genes in the presence of AC1-004 were determined by RT-PCR. (C) In vitro tube formation assay. The wells of a 96-well plate were coated with ice-cold BD Matrigel™ matrix gel solution. Human umbilical vein endothelial cells (HUVECs) were seeded and tubule branches were photographed after 16 h of incubation. Suramin was used as a positive control.

Effects on HIF-1a accumulation under hypoxia

Inhibition of HRE activation under hypoxia can be explained by either the reduction in the expression level of HIF-1α or the interference of HIF-1α binding to HRE. The effect of AC1-004 on HIF-1α expressions in various human carcinoma cancer cell lines (i.e., SK-HEP1, MDA-MB-435, HCT-116, and Caki-1) was determined by Western blot analysis (Fig. 2A). HIF-1α expression in normoxia was not sufficiently high to be detected. Under hypoxia, the HIF-1α expression level was significantly increased, indicating that the HIF-1α protein was accumulated due to the inactivation of prolyl hydroxylase activity. When the cells were incubated in the presence of AC1-004, the accumulation of HIF-1α was inhibited in a dose-dependent manner (Fig. 2A). This result suggests that AC1-004 inhibited HRE activation under hypoxia, via a reduction in HIF-1α protein expression.

Effect on HIF-1 target gene expression as an angiogenesis inhibitor

The inhibitory effect of AC1-004 on HIF-1α accumulation was analyzed via the expression of the downstream targets VEGF and EPO, which are associated with the angiogenesis of an aggressive tumor. In the presence of AC1-004, the mRNA levels of VEGF and EPO in HCT-116 cells decreased in a dose-dependent manner, while the mRNA expression level of HIF-1α was not affected (Fig. 2B). This result indicates that AC1-004 suppressed the expression of VEGF and EPO at the transcription level, further suggesting that AC1-004 is associated with the inhibition of angiogenesis.

In vitro tube formation, an assay for the capillary formation of HUVECs, is a simple but powerful tool for examining angiogenetic associations. HUVECs were grown to form capillary-like structures on the Matrigel™ surface, as shown in Fig. 2C. Suramin, an angiogenesis inhibitor [32,33], destroyed the capillary-like structures on the Matrigel™, as expected. When HUVECs were incubated in the presence of AC1-004 at 10 and 30 μM, respectively, capillary-like structure was not formed indicating that AC1-004 inhibited in vitro tube formation of HUVEC on matrigel by inhibiting VEGF function via blocking accumulation of HIF-1α. When cytotoxic effect of AC1-004 on HUVECs was also examined using the MTT assay, the growth of HUVECs was not affected by AC1-004, up to 30 μM (Supplementary material). This result implies that AC1-004 may have an inhibitory effect on angiogenesis.

AC1-004 regulates stability of HIF-1α through Hsp90 and Akt signaling

To address the mechanism of AC1-004 in inhibiting HIF-1α activity, changes in HIF-1α mRNA levels were investigated during AC1-004 treatment. The HIF-1α mRNA level was steady in the presence of AC1-004 (Fig. 2B), indicating that the inhibition of HIF-1α accumulation was not regulated at the transcription level. In order to further evaluate the inhibitory activity of AC1-004 on HIF-1α accumulation, its effect on the stability of HIF-1α protein was examined in HCT-116 cells pretreated with proteasomal inhibitor MG132 (Z-Leu-Leu-Leu-al) as shown in Fig. 3A. Pretreatment of cells with MG132 resulted in an accumulation of HIF-1α in normoxia, as expected. Furthermore, the inhibition of HIF-1α accumulation by AC1-004 was reversed in the presence of MG132, indicating that AC1-004 may affect HIF-1α protein stability via proteasomal-dependent degradation.

It has been reported that inhibition of PI3K promoted degradation of HIF-1α indirectly by reducing steady state concentrations of Hsp90 [34]. To address whether or not AC1-004 affects inhibition of Hsp90, the expression levels of Cyclin D1 and CDK4, client proteins of Hsp90, were investigated in the presence of AC1-004 and compared with that in the presence of geldanamycin (GA), a well-known Hsp90 inhibitor [35]. As described in Fig. 3B, it was found that GA decreased the expressions of cyclin D1 and CDK4. Upon the treatment with AC1-004, expression levels of both Cyclin D1 and CDK4 were reduced. Since Hsp90 interacts with Akt and disruption of Hsp90 function inhibits phosphorylation of Akt [36,37], involvement of AC1-004 in PI3K/Akt signaling pathway was also examined. LY294002, a PI3K inhibitor, lowered steady-state level of Hsp90. It also induced dephosphorylation of Akt and GSK3β as described in Fig. 3C. When cells were treated with AC1-004 at 30 μM under hypoxia, expression level of Hsp90 was reduced and phosphorylations of both Akt and GSK3β were down-regulated, which indicates involvement of AC1-004 in regulation of PI3K/Akt signaling pathway. This result suggests that AC1-

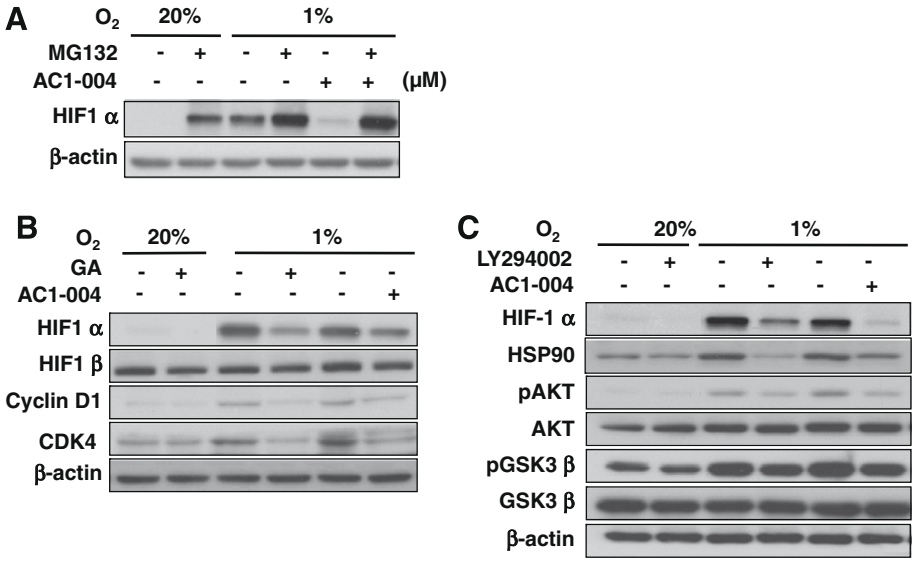

Fig. 3. AC1-004 regulates stability of HIF-1α, via Hsp90 and Akt signaling. (A) The effect of AC1-004 on the stability of HIF-1α protein was examined in HCT-116 cells pretreated with proteasomal inhibitor MG132 (Z-Leu-Leu-Leu-al) for 4 h and treatment with AC1-004 for a subsequent 12 h. Western blot analysis was carried out with samples prepared from the aforementioned cells. (B) The effect of AC1-004 on Hsp90 client genes. Expression levels of Cyclin D1 and CDK4 were examined in the cells treated with geldanamycin (GA) or AC1-004. (C) The effect of AC1-004 on the PI3K-Akt signaling pathway. Protein expression level of Hsp90 and the phosphorylation of Akt and GSK3β were examined in the cells treated with the PI3K inhibitor LY294002, or with AC1-004.

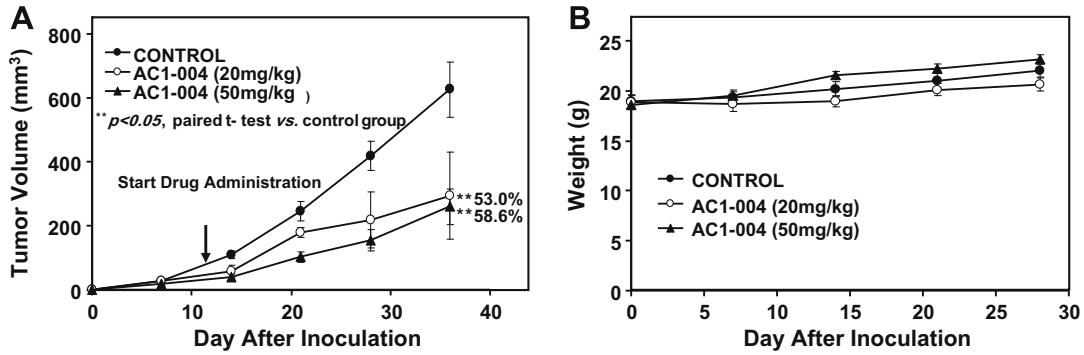

Fig. 4. In vivo antitumor activity of AC1-004. The MDA-MB-435 cell line was used to generate tumors in nude mice. When the tumor volume reached ~100 mm^2, tumor-bearing mice were treated with 20 and 50 mg/kg of AC1-004 every other day for 5 weeks. (A) Tumor growth inhibition by AC1-004. (B) Changes in body weight.

004 regulates the stability of HIF-1α via the Hsp90-Akt pathway, resulting in the degradation of HIF-1α. Further study is required, however, to elucidate the mechanism of AC1-004 in greater detail.

AC1-004 inhibited the tumor growth of MBA-MB-435 cells in a mouse model

The in vivo antitumor activity of AC1-004 was evaluated using athymic nude mice and the human cancer cell line MDA-MB-435. When the tumor volume reached ~100 mm^2, tumor-bearing mice were treated with AC1-004 (intraperitoneally) every other day until the end of the study (Fig. 4). The administration of AC1-004 to the mice at doses of 20 and 50 mg/kg significantly inhibited tumor growth—up to 53% and 58.6%, respectively—compared to the vehicle-treated control group. AC1-004 did not cause any side effects, such as skin ulcers or other severe symptoms. This result further supports the potential development of AC1-004 as a novel HIF inhibitor for cancer therapy.

Conclusions

In this report, we described the discovery of a novel benzimidazole AC1-004 (4) as a potent HIF-1α inhibitor. AC1-004 exhibited an IC_{50} value of 1.8 μM in a cell-based HRE-dependent reporter assay using HCT-116 cells. In particular, AC1-004 significantly suppressed the hypoxia-induced mRNA levels of VEGF and EPO, as well as in vitro tube formation on the Matrigel™, indicating its potential inhibitory effect on angiogenesis. The inhibitory effect of AC1-004 on HIF-1α accumulation may be explained by the disruption of the Akt signaling pathway through Hsp90, leading to a destabilization of HIF-1α protein. In vivo antitumor effect of AC1-004, along with its inhibitory effect on angiogenesis, suggests that AC1-004 is a useful lead compound for development of cancer therapy. The detailed mode of action of AC1-004, however, remains to be clarified.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.05.022.

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