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Bafilomycin induces the p21-mediated growth inhibition of cancer cells under hypoxic conditions by expressing HIF-1α.

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A list of nonstandard abbreviations: HIF-1α, hypoxia-inducible factor 1alpha; BM, bafilomycin A1; CM, concanamycin A; ARNT, aryl hydrocarbon nuclear receptor translocator; pVHL, Von Hippel-Lindau protein; PHDs, HIF-1-prolyl hydroxylases; FIH, factor-inhibiting HIF; VEGF, vascular endothelial factor; PGK, phosphoglycerokinase; RT-PCR, reverse transcription polymerase chain reaction.

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ABSTRACT

Bafilomycin A1, a macrolide antibiotic isolated from *Streptomyces* sp, has been used as an inhibitor of vacuolar H+ ATPase. Bafilomycin has been also evaluated as a potential anticancer agent since it inhibits cell proliferation and tumor growth. Although these anticancer effects of bafilomycin are considered to be attributable to the intracellular acidosis by V-ATPase inhibition, the exact mechanism remains unclear. In the present study, we tested the possibility that bafilomycin targets a tumor-promoting factor, hypoxia-inducible factor-1α (HIF-1α). Bafilomycin A1 and its analogue, concanamycin A, were found to up-regulate HIF-1α in 8 human cancer cell-lines, and this effect is attributed to inhibited degradation of HIF-1α protein. Furthermore, the HIF-1α induction by bafilomycin was augmented by hypoxia, which caused a robust induction of p21 and cell cycle arrest in cancer cells. The cell cycle inhibition was shown only in cancer cells expressing both HIF-1α and p21. In HIF-1α(+/+) or HIF-1α(-/-) fibrosarcomas grafted in nude mice, bafilomycin showed the HIF-1α-dependent anticancer effect. Based on these results, the exorbitant expression of HIF-1α is likely to contribute to the anticancer action of bafilomycin.

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INTRODUCTION

Bafilomycin A1, a macrolide antibiotic isolated from *Streptomyces* sp, has been used as an inhibitor of vacuolar H+ ATPase (V-ATPase). It binds to a pocket formed by V0 sector subunit c (ATP6V0C) of the V-ATPase complex, and inhibits H^+ translocation by preventing the rotation of the ATP6V0C multimer, which causes the accumulation of H^+ in the cytoplasm (Bowman et al., 2004). Biologically, it has been found that bafilomycin induces cell growth inhibition (Ohkuma et al., 1993) and apoptosis (Kinoshita et al., 1996, Nakashima et al., 2003). *In vivo*, bafilomycin also inhibits the growth of xenografted tumors (Ohta et al., 1998), and thus could be evaluated as a potential anticancer agent. These biological effects of bafilomycin are considered to be attributable to the intracellular acidosis by V-ATPase inhibition. Is the acidosis only one mechanism underlying the anticancer activity of bafilomycin? Generally, the agents that have *in vivo* anticancer activities inhibit some cancer-specific events, but the acidosis by bafilomycin occurs in normal cells as well. Therefore, we hypothesized that bafilomycin targets a cancer-specific molecule.

Hypoxia-inducible factor-1α (HIF-1α) is a basic-helix–loop–helix protein of the PAS family (Wang et al., 1995). It plays a key role in cellular adaptation to hypoxia by up-regulating 60 or more genes essential for angiogenesis and cell survival. To date, the roles of HIF-1α in tumor progression have been extensively investigated and its over-expression is frequently found in various human tumors (Zhong et al., 1999). HIF-1α levels in tumors are positively related with tumor hypervascularity, aggressiveness, and poor prognosis (Birner et al., 2000, Zagzag et al., 2000). Moreover, xenograft studies have disclosed that HIF-1α is essential for tumor growth and angiogenesis (Ryan et al., 2000). Therefore, HIF-1α is indicated as an aggravating factor in cancer diseases. However, in stark contrast with the above, HIF-1 α overexpression in hypoxic cancer cells has been suggested to inhibit cell division by inducing p21, p27, or p53, and to promote apoptosis by inducing p53, Nip3, Noxa, or HGTD-P (Goda et al., 2003; Bacon and Harris, 2004). Thus, HIF-1α appears to be a double-edged sword from the viewpoint of tumors subjected to hypoxia, and thus its expression is probably controlled at optimal levels in growing tumors. In this respect, both HIF-1α suppression and overexpression could inhibit the growth of hypoxic tumors.

In the present study, we address the mechanism underlying the anticancer action of bafilomycin. Bafilomycin A1 and its analogue, concanamycin A, were found to induce HIF-1α in 8 human cancer cell-lines. The HIF-1α induction by bafilomycin was augmented by hypoxia, which caused a robust induction of p21 and cell cycle arrest in cancer cells. In fibrosarcoma xenografts, bafilomycin showed the HIF-1α-dependent anticancer effect. The exorbitant expression of HIF-1α is likely to contribute to the anticancer action of bafilomycin.

MATERIALS AND METHODS

Materials. Bafilomycin A1 and MG132 were purchased from Alexis Biochemicals (Lausen, Switzerland), and concanamycin A, cycloheximide, propidium iodide, and other chemicals from Sigma-Aldrich (St. Louis, MO). Culture media and fetal calf serum were purchased from GIBCO/BRL (Grand Island, NY). Anti-HIF-1α antiserum was generated in rabbits against a bacterially expressed fragment encompassing amino acids 418-698 of human HIF-1α, as previously described (Chun et al., 2001). p53, p27, mouse p21, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human p21, Hemagglutinin, pVHL and Flag antibodies were purchased from Cell Signaling (Beverly, MA), Roche (Basel, Switzerland), Pharmingen (San Diego, CA) and Sigma-Aldrich, respectively.

Cell culture. PC3 (prostate), Caki-1 (kidney), SiHa (uterine cervix), and Hep3B (liver) cancer cell-lines were obtained from ATCC (Manassas, VA). Four lung cancer cell-lines, HCC1171 (epithelial origin), HCC2108 (epithelial), HCC1195 (squamous), and HCC2279 (squamous) were obtained from the Korean Cell Line Bank (Seoul), and a HIF-1α-null MEF cell-line was provided as described previously (Ryan et al., 2000). HCT116(p21-/-) and HCT116(p21+/+) were generous gifts from Dr. Deug Y Shin (Dankook University College of Medicine). Lung cancer cells were cultured in RPMI 1640 medium and other cells in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, in a 5% CO_2 humidified atmosphere at 37°C. O_2 levels in the chamber were either 20% (normoxic) or 1%

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(hypoxic). Bafilomycin A1 or concanamycin A were administered to medium 5 minutes prior to normoxic or hypoxic incubation for 4 hours.

Immunoblotting and Immunoprecipitation. To quantify protein levels, total cell lysates were prepared as described previously (Chun et al., 2001). Proteins were separated on SDS/polyacrylamide gels, and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk and incubated overnight at 4°C with a primary antibody diluted 1:1000. Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (diluted 1:5000) for 2 hours, and antigen-antibody complexes were visualized using an Enhanced Chemiluminescence Plus kit (Amersham Biosciences Corp., Piscataway, NJ). β-actin protein was used as an internal standard. For co-immunoprecipitation of HIF-1α and pVHL, cell lysates (150 μg of protein) were incubated with 10 μl of anti-HIF-1α antiserum or preimmune rabbit serum at 4°C for 4 hours. The immune complex was further incubated with protein A/G-Sepharose beads (Amersham Biosciences Corp.) at 4°C for 4 hours. The antigen-bead complexes obtained were washed extensively with lysis buffer. Immunocomplexes were eluted by boiling for 3 minutes in a sample buffer containing 2% SDS and 10 mM dithiothreitol, subjected to SDS-PAGE, and then immunoblotted using anti-HIF-1α or anti-pVHL antibody.

Semiquantitative RT-PCR. To quantify mRNA levels, we used a highly sensitive, semi-quantitative RT-PCR methods, as previously described (Yeo et al., 2003). Total RNAs were isolated from cultured cells or rat kidney tissues using TRIZOL (GIBCO/BRL). One μg of RNA was reverse-transcribed and the cDNA obtained was amplified over 18 PCR cycles in a reaction mixture containing 5 μCi [α-^{32}P]dCTP and 250 nM of each primer set. PCR products were electrophoresed on a 4% polyacrylamide gel, and dried gels were autoradiographed. Primers for human HIF-1α, VEGF, PGK 1, enolase 1, and β-actin were constructed as previously described (Yeo et al., 2003).

Construction and assay of a p21 reporter gene. To make a p21 promoter-driven luciferase reporter gene, the DNA coding human p21 promoter region (-966 to -2) containing a c-Myc binding site

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was cloned using PCR, and then inserted into the *Kpn*I and *Bgl*II sites of the pGL3 promoter plasmid (Promega). PC3 cells were co-transfected with 0.5 μg each of reporter gene and plasmid cytomegalovirus-β-gal using Lipofectamin (Invitrogen). After being allowed to stabilize for 48 h, cells were lysed to determine luciferase and β-gal activities. Luciferase activities were analyzed using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany), and β-gal assays were performed to normalize transfection efficiencies.

Chromatin immunoprecipitation. Cells were fixed with formaldehyde, and soluble chromatin samples were immunoprecipitated with anti-c-Myc or anti-HIF-1α at 4°C overnight (Jung *et al*, 2005). DNA isolated from immunoprecipitated material was amplified by semiquantitative PCR with [α-^{32}P]dCTP. The PCR primer sequences used were 5'-GATTTGTGGCTCACTTCGT-3' and 5'-GCTCCACAAGGAACTGACT-3', which produced a 320 bp fragment including the c-Myc binding site of the p21 gene. PCR products were electrophoresed in a 4% polyacrylamide gel, and dried gels were autoradiographed.

Cell cycle analysis. PC3 cells were plated in 10 cm culture dishes at concentrations determined to yield 70-80% confluence within 24 h. Cells were then incubated under normoxic or hypoxic conditions in the absence or presence of bafilomycin A1 for 16 hours. Cells were then harvested and resuspended in 200 μl of PBS and fixed in 75% ethanol for 30 min on ice. After washing with PBS, cells were labeled with propidium iodide (0.05 mg/ml) in the presence of RNase A (0.5 mg/ml) and then incubated in the dark for 30 min. DNA contents were analyzed using a Becton Dickinson FACStar flow cytometer. Propidium iodide was excited using an argon laser at 488 nm and detected at 630 nm.

Animals and tumor grafts. Male nude (BALB/cAnNCrj–nu/nu) mice were purchased from Charles River Japan Inc. (Shin-Yokohama, JAPAN). Animals were housed in a specific pathogen-free room under controlled temperature and humidity. All animal procedures were performed according with the procedures in the Seoul National University Laboratory Animal Maintenance Manual. Mice aged 7–8

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weeks were injected subcutaneously in the flank with $5x10^6$ viable cells of HIF-1α (+/+) or HIF-1α (-/-) MEF. Tumor volumes were measured with a caliper and calculated using the formula: Volume = $axb^2/2$, where **a** was the width at the widest point of the tumor and **b** was the maximal width perpendicular to **a**.

Tumor histology and TUNEL staining. Tumors were removed 4 days after injecting bafilomycin A1, fixed with formalin, and embedded in paraffin. Serial sections (6 μm thick) were cut from each paraffin block, and stained with hematoxylin and eosin (H&E). Necrosis was identified at a magnification of 40X, and examined using a Sony XC-77 CCD camera and a Microcomputer Imaging Device model 4 (MCID-M4) image analysis system. The extent of necrosis was determined in four different cross sections per tumor by dividing the total cross-sectional necrotic area by the total cross-sectional area. An ApopTag *in situ* apoptosis detection kit of Oncor (Gaithersburg, MD) was used to evaluate apoptotic death. Serial sections were dewaxed, treated with proteinase K, incubated with equilibration buffer for 10 min, and then incubated with working-strength TdT enzyme solution at 37°C for 2 h. The reaction was terminated by incubation in working-strength stop/wash buffer for 30 min at 37°C. Sections were then incubated with anti-digoxigenin peroxidase, and then with diaminobenzidine and 0.01% H_2O_2 for 5 min at room temperature. Finally, they were lightly counterstained with H&E and examined under an optical microscope.

Statistical analysis. All data were analyzed using Microsoft Excel 2000, and results are expressed as means and standard deviations. The Mann-Whitney U test (SPSS 10.0 for Windows, Chicago, IL) was used to compare reporter activities or protein levels in cultured cells. Tumor volumes of the control and the bafilomycin-treated groups were compared using analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered statistically significant at the $P < 0.05$ level. All statistical tests were two-sided.

RESULTS

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HIF-1α is induced by bafilomycin A1 or concanamycin A. In 8 cancer cell-lines, HIF-1α expression was increased in a dose-dependent manner by bafilomycin A1 (Fig. 1A, left column), whereas β-actin expression was not (data not shown). We examined the effect of another V-ATPase inhibitor, concanamycin A (Forgac et al., 1989), on HIF-1α expression, and found that it also induced HIF-1α (Fig. 1A, right column). HIF-1α expression in PC3 cells was increased in a time-dependent manner by bafilomycin A1 (Fig. 1B). When PC3 cells were incubated under hypoxic conditions, HIF-1α levels were synergistically increased by bafilomycin (Fig. 1C). To clarify the mechanism of HIF-1α induction by bafolomycin, we examined HIF-1α expression at the mRNA and protein levels. BM did not affect HIF-1α mRNA levels (Fig. 2A), but inhibited HIF-1α degradation to extend protein half-life by 4-fold (Fig. 2B). Proteasomal degradation of HIF-1α is preceded by the binding of Von Hippel-Lindau tumor suppressor protein (pVHL) and HIF-1α. We thus examined the effect of bafilomycin on the pVHL-HIF-1α interaction using immunoprecipitation, and found that bafilomycin strongly inhibited the pVHL binding to HIF-1α. Bafilomycin seems to deregulate the pVHL-dependent HIF-1α degradation process. Recently, it has been reported that HIF-1α can be stabilized under acidic conditions by nuclear sequestration of pVHL (Mekhail et al. 2004). Since the inhibition of V-ATPase could acidify the cytoplasm, we tested the possibility that this pH-dependent regulation of HIF-1α is responsible for the bafilomycin effect. However, the bafilomycin effect on HIF-1α was not attenuated in alkaline media (Fig. 2D). After treatment of bafilomycin, pVHL was not sequestered to the nucleus, while HIF-1α was present specifically to the nucleus (Fig. 2E). Moreover, an acridine orange staining showed that endosomal pH was not changed by 4 hour-treatment with 10 nM BM (supplemental result 1). These results suggest that the HIF-1α stabilization by bafilomycin is irrelevant to the pH-dependent regulation.

Bafilomycin induces p21 expression. HIF-1α is known to have two different functions; hypoxic adaptation and growth arrest (summarized Fig. 3A). To determine the significance of bafilomycin-induced HIF-1α expression, we first analyzed mRNA levels of VEGF, phosphoglycerokinase 1 and enolase 1. However, these mRNAs were not increased significantly by bafilomycin, while they were up-

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regulated under hypoxic conditions (Fig. 3B). Moreover, bafilomycin did not enhance the transcriptional activity of HIF-1α in an erythropoietin enhancer-luciferase reporter system (data not shown). This suggests that HIF-1α induced by bafilomycin is less active than that by hypoxia. We next examined the expressions of cell cycle inhibitors p21, p27, and p53. In PC3 cells, p21(cip1/waf1) expression was increased synergistically by bafilomycin and hypoxia in combination (Fig. 3C). However, p27 was not induced by bafilomycin and p53 was not expressed in PC3 cells. Moreover, in 4 cancer cell-lines, bafilomycin induced p21 in a dose-dependent manner, but did not affect p53 expression (Fig. 3D). To examine whether bafilomycin stimulates p21 promoter activity, we made a luciferase reporter plasmid containing the p21 promoter. Fig. 3E shows that bafilomycin enhanced p21 promoter activity in a dose-dependent manner (left panel) and further increased it under hypoxic conditions (right panel). Regarding the mechanism of p21 induction by HIF-1α, Koshiji et al. (2004) has demonstrated that HIF-1α dissociates c-Myc (a repressor) from p21 promoter, and thereby de-represses p21. Therefore, we examined whether bafilomycin inhibits the c-Myc binding to p21 promoter via HIF-1α, and found that in bafilomycin-treated cells, the c-Myc binding noticeably decreased but the HIF-1α binding increased (Fig. 3F). This suggests that bafilomycin-induced HIF-1α counteracts c-Myc-mediated repression of p21 and expresses p21.

HIF-1α is essential for bafilomycin-induced p21 expression and cell cycle arrest. Since p21 functions as a cell cycle inhibitor, we examined whether bafilomycin induces cell cycle arrest via p21. As expected, the G1 population of PC3 cells was increased synergistically by bafilomycin and hypoxia in combination (Fig. 4). To confirm that HIF-1α mediates bafilomycin-induced p21 expression and cell cycle arrest, we examined the effects of bafilomycin in HIF-1α (+/+) and HIF-1α (-/-) mouse embryonic fibroblast (MEF) cells. HIF-1α (+/+) cells showed a good response to bafilomycin in HIF-1α expression, but HIF-1α (-/-) cells failed to express HIF-1α (Fig. 5A). Interestingly, p21 induction and G1 arrest did not occur in HIF-1α (-/-) cells, whereas both events were well observed in HIF-1α (+/+) cells (Fig. 5B and 5C). To examine whether p21 mediates the bafilomycin-induced cell cycle arrest, HCT116 cells were treated with bafilomycin. HIF-1α levels in both p21 (+/+) and p21 (-/-) cells were synergistically

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enhanced by bafilomycin and hypoxia, but p21 induction was observed only in p21 (+/+) cells (Fig. 6A). Although HIF-1α was induced in both cell-lines, bafilomycin did not arrest the cell cycle in p21 (-/-) cells under hypoxic conditions, whereas it did in p21 (+/+) cells (Fig. 6B).

HIF-1α is required for the anticancer action of bafilomycin. The HIF-1α-dependent inhibition of cell proliferation could be a good strategy for solid tumor treatment because HIF-1α overexpression is a typical feature of tumors subjected to hypoxia or with oncogene or tumor suppressor gene mutations (Semenza, 2003). Thus we examined *in vivo* the relation between the anticancer activity of bafilomycin and HIF-1α expression. Mice grafted with HIF-1α (+/+) or HIF-1α (-/-) fibrosarcomas were treated with a single peritoneal injection of bafilomycin. The upper panels in Fig. 7A and 7B illustrate the gross morphologies of the tumors which were excised from the flanks of mice 4 days after vehicle or bafilomycin treatment. HIF-1α (+/+) tumors in mice treated with bafilomycin were much smaller than those in animals treated with vehicle only. Fig. 7A shows the growth rate of the HIF-1α (+/+) tumor, plotted as average tumor volume versus time. Bafilomycin inhibited tumor growth significantly. However, bafilomycin failed to inhibit the growth of HIF-1α (-/-) tumor (Fig. 7B). These results indicate that HIF-1α expression is responsible for the anticancer effect of bafilomycin. In tumor histology, bafilomycin-treated HIF-1α (+/+) tumors showed disorganized cell layers and condensed chromatin (Fig. 7C), and frequent TUNEL-positive nuclei (Fig. 7D). Necrotic areas were markedly enhanced in bafilomycin-treated HIF-1α (+/+) tumors (Fig. 7E). We also confirmed that bafilomycin induced the expressions of HIF-1α and p21 in the tumors (Fig. 7F). However, none of these bafilomycin effects were observed in HIF-1α (-/-) tumors.

DISCUSSION

In this study, we found that bafilomycin increased HIF-1α. HIF-1α induced by bafilomycin arrested the cell cycle in the G1 phase by inducing p21, which is believed to be responsible for the anticancer effect of bafilomycin *in vivo*. In this work, we emphasize that the anticancer effect of bafilomycin *in vivo*

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seems likely to depend on exorbitant expression of HIF-1α in hypoxia and this effect could provide a potential therapeutic strategy for solid tumor having hypoxic region.

HIF-1α has been suggested to act as both a positive and a negative regulator of tumor growth. Many xenograft experiments using HIF-1α null tumors (Ryan et al., 2000) and HIF-1α inhibitors (Yeo et al., 2003) have demonstrated that HIF-1α is associated with tumor growth. On the contrary, Carmeliet et al. (1998) reported that HIF-1α null tumors grew unexpectedly rapidly even though tumor vessel formation was impaired. They also found that cancer cells in HIF-1α (-/-) tumors were more proliferative and less apoptotic than those in HIF-1α (+/+) tumors. Mack et al. (2003) also demonstrated that HIF-1α stabilization by VHL gene knock-out decreased, rather than increased, tumor growth, despite the induction of HIF-1 target genes. Regarding this controversy surrounding the role of HIF-1α in tumor growth, a recent report may provide an answer (Blouw et al., 2003). HIF-1α (-/-) astrocytoma was found to grow slower in vessel-poor subcutaneous spaces with poor vascularity than in vessel-rich brain parenchyma. Thus, the differential roles of HIF-1α on tumor growth seem to depend on the extant microenvironment of the tumor. If so, under some circumstances, HIF-1α overexpression might be a better strategy for cancer therapy than its inhibition, and hence the attractiveness of bafilomycin as a HIF-1α targeting anticancer drug. Moreover, 'tumor-promoting effect' of HIF-1α predominantly depends on its transcriptional activity, whereas 'tumor-inhibiting effect' of HIF-1α does not. Since bafilomycin treatment only induced the expression of HIF-1α with low transcriptional activity, it could specifically enhance the 'tumor-inhibiting effect'. Therefore, bafilomycin may be a candidate for anticancer agent by exorbitantly inducing HIF-1α in hypoxic tumors.

Although bafilomycin induced HIF-1α levels under normoxic conditions, it has little effect on the expressions of HIF-1 targeted genes. This effect of Bafilomycin on HIF-1α could be suspected of the factor-inhibiting HIF (FIH). In the absence of a hypoxic signal, HIF-1α can be inactivated by factor-inhibiting HIF (FIH). FIH hydroxylates an asparagine residue (N803) within the transactivation domain of HIF-1α, which blocks its recruitment of p300 co-activator (Lando et al., 2002). Moreover, under hypoxic conditions, asparagine hydroxylation is inhibited due to limited oxygen, and HIF-1α remains

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unmodified and activated. This appears to be one of the reasons why HIF-1α induced by bafilomycin has little transcriptional activity; similarly, HIF-1α induced by heat shock was found to have no transcriptional activity (Katschinski et al., 2002). However, HIF-1α induced by bafilomycin induced p21 transcription. Recently, Koshiji et al. (2004) demonstrated the mechanism of p21 induction by HIF-1α, i.e., c-Myc represses p21 transcription; HIF-1α displaces c-Myc binding from p21 promoter, and thereby de-represses p21. They also demonstrated that the N-terminal of HIF-1α without the transactivation domain, is sufficient to antagonize c-Myc. Therefore, HIF-1α induced by bafilomycin can induce p21 expression, despite a low transcriptional activity.

Since c-Myc also represses p27 transcription by direct binding of Myc/Max complexes in p27 promoter, HIF-1α can de-repress p27 by counteracting c-Myc, as it does p21 (Gartel and Shchors, 2003). Indeed, several reports demonstrated that HIF-1α mediates the hypoxic induction of p27 (Goda et al., 2003; Mack et al., 2005). If so, p27 could be induced by bafilomycin treatment, but it was not induced in our experimental settings. We think this discrepancy could result from differences in p21 and p27 responses to HIF-1α; p27 responds to HIF-1α less sensitively than p21, which can be supported by the following reports. After 48 hour hypoxia, p21 mRNA levels increased by 4-fold whereas p27 mRNA levels increased by about 90% in HIF-1α (+/+) B cells (Goda et al., 2003). Similarly, Mack et al. (2005) also demonstrated that p21 transcription was induced by 9-fold in hypoxia but p27 transcription was induced by 1-fold. Moreover, p21 mRNA levels of VHL (-/-) MEF (overexpressing HIF-1α) markedly increased 140-fold versus those of VHL (+/+) MEF, whereas p27 mRNA levels increased just 9-fold. In the present study, we also found that p27 was induced by expressed HIF-1α less sensitively than p21 (supplemental result 2). Based on these results, it is speculated that the amount of bafilomycin-induced HIF-1α is enough for p21 induction, but not for p27 induction.

The *in vivo* effect of bafilomycin on tumor growth has previously been evaluated in xenografted pancreatic tumors (Ohta et al., 1998). Bafilomycin A1 (1 mg/kg) was injected once daily into nude mice from when tumors reached about 50 mm^3 in size. However, they did not observe a significant retardation in tumor growth until tumors reached 300 mm^3, which required 21 injections. However, when we injected mice having fibrosarcoma with 1 mg/kg of bafilomycin A1, we observed its inhibitory effect on

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tumor growth the following day. In view of the fact that the cell proliferation inhibition by bafilomycin depends on HIF-1α levels, and that this is synergistically enhanced by hypoxia, it is believed that bafilomycin can inhibit the growth of large tumors subjected to hypoxia more effectively than that of small tumors, which explains its dramatic effect on 400-500 mm^3 sized tumors. If so, bafilomycin may be a useful therapeutic agent for large solid tumors.

HIF-1α is tightly regulated by oxygen tension. Under aerobic conditions, PHD prolyl hydroxylases hydroxylate P402 and P564 of HIF-1α using molecular oxygen, and then the pVHL-E3 ubiquitin ligase complex binds to the hydroxylated HIF-1α, which results in ubiquitination and proteasomal degradation of HIF-1α (Epstein, 2001; Jaakkola, 2001). Under hypoxic conditions, however, HIF-1α hydroxylation is inhibited due to the limitation of oxygen, thereby precluding the binding of pVHL and stabilizing HIF-1α. In addition, a recent report demonstrated that pVHL was localized to the nucleoli under acidic (~pH 6.0) conditions, whereas it was present diffusely in the nucleus and the cytoplasm under neutral pH conditions (Mekhail et al. 2004). They also found that the nucleolar localization of pVHL restricted the pVHL binding to HIF-1α and caused the stabilization of HIF-1α. Considering the acidifying effect of bafilomycin, it is plasusible that HIF-1α stabilization by bafilomycin is attributable to intracellular acidosis and nucleolar sequestration of pVHL. However, the HIF-1α stabilization by bafilomycin occurred even under alkaline pH conditions, and pVHL was not redistributed by bafilomycin. Furthermore, we found that the endosomal pH was not noticeably changed by 10 nM bafilomycin, whereas it was by 100 nM bafilomycin. Intracellular pH is unlikely to be changed by treating cells with bafilomycin at a low concentration (10 nM) and for a short time (4 h). Therefore, these results suggest that bafilomycin stabilize HIF-1α via other mechanism rather than via acidification. The mechanism underlying bafilomycin-induced HIF-1α stabilization remains to be investigated in the next study.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Bafilomycin and concanamycin induce HIF-1α. (A) Dose-response of HIF-1α expression. Eight cancer cell-lines were incubated with bafilomycin A1 (BM) or concanamycin A (CM) for 4 hours and harvested. The concentrations of BM and CM used were 10, 5, 2, 1, and 0.2 nM. Protein levels of HIF-1α were evaluated by immunoblotting. (B) Time course of HIF-1α expression. PC3 cells were incubated with 10 nM BM for the times indicated above the figure. (C) Synergistic induction of HIF-1α by bafilomycin and hypoxia. PC3 cells were incubated under normoxic or hypoxic conditions in the absence or in the presence of 10 nM BM for 4 hours. HIF-1α levels were evaluated by immunoblotting.

Fig. 2. Bafilomycin stabilizes HIF-1α protein. (A) HIF-1α mRNA levels. Total RNAs were extracted from PC3 cells treated with 10, 5, 2, 1, or 0.2 nM BM, and HIF-1α and β-actin mRNAs were analyzed by semiquantitative RT-PCR. (B) HIF-1α stability. PC3 cells were incubated in the absence (- BM) or in the presence (+ BM) of 10 nM bafilomycin A1 for 4 hours and then treated with 60 μg/ml of cycloheximide (CHX). After 5, 10, 30, 60, or 120 minutes, the cell lysates were analyzed by immunoblotting using anti-HIF-1α and anti-β-actin antibodies (upper panel). Band intensities were quantified using a Microcomputer Imaging Device model 4 (MCID-M4) image analysis system, and are plotted in lower panel. Half-lives ($t_{1/2}$) were calculated from the slopes of first-order decay curves. Each point represents the mean value of three separate experiments. (C) Bafilomycin dissociates pVHL from HIF-1α. PC3 cells were treated with BM and MG132 (10 μM) for 6 hours. HIF-1α was immunoprecipitated using anti-HIF-1α antibody or non-immunized rabbit serum (Pre), and the co-precipitation of pVHL with HIF-1α was analyzed with anti-pVHL antibody. (D) HIF-1α expression under alkaline conditions. A HEPES buffer (50 mM, pH 7.2-8.0) was added to buffer-free DMEM, supplemented with 5 mM $NaHCO_3$ and 10% fetal calf serum. Cells were incubated in the alkaline media for 30 minutes and treated with 10 nM BM for 4 hours. MG, treatment of 10 μM MG132 proteasome inhibitor. (E) Sub-localization of pVHL and HIF-1α. After PC3 cells were treated with BM (10, 2, 1 nM)

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for 4 hours, cells were harvested and fractionated to total (*T*), cytosolic (*C*), and nuclear (*N*) fractions. HIF-1α and pVHL proteins were analyzed in each fraction using anti-HIF-1α and anti-pVHL antibodies.

Fig. 3. Bafilomycin induces p21 expression. (A) Two different roles of HIF-1α. (B) PC3 cells were incubated under normoxic or hypoxic conditions for 16 hours, in the absence or in the presence of 10 nM of BM. Expression of genes related to angiogenesis and glycolysis. mRNA levels of vascular endothelial growth factor (VEGF), phosphoglycerate kinase 1 (PGK), enolase 1 (Eno), and β-actin were determined by semiquantitative RT-PCR. C, normoxic control; H, hypoxia; BM, BM treatment; H+BM, BM treatment under hypoxic conditions. (C) Synergistic induction of p21 by BM and hypoxia. In PC3 cells, p27, and β-actin levels were analyzed by immunoblotting. Band intensities of p21 were quantified using the MCID-M4 image analysis system and are plotted in the right panel. Each point represents the mean and standard deviation of four separate experiments. *: $p<0.05$ *vs.* the normoxic control. (D) Bafilomycin-induced p21 expression in various cell-lines. Four cancer cell-lines were incubated with various concentrations (10, 5, 2, 1, or 0.2 nM) of BM for 4 hours, and then harvested for p21, p53 and β-actin immunoblotting. (E) Bafilomycin activates the p21 promoter. PC3 cells were transfected with a p21-promoter reporter plasmid, and treated with BM for 4 hours. Luciferase activities are quoted as relative values versus the control value, and are plotted as the means+SDs of 4 experiments. *: $p < 0.05$ *vs.* the control. (F) Bafilomycin-induced HIF-1α dissociates c-Myc from p21 promoter. After PC3 cells were treated with 10 nM bafilomycin for 16 hours, c-Myc or HIF-1α binding to p21 promoter was analyzed using chromatin immunoprecipitation. Proximal promoter DNAs of p21 were amplified by 32 cycles of PCR, electrophoresed, and autoradiographed.

Fig. 4. Bafilomycin induces G_1 arrest under hypoxic conditions. PC3 cells were incubated under normoxic or hypoxic conditions for 16 hours, in the absence or in the presence of 10 nM of BM. Cell cycle analysis was performed as described in Methods. C, normoxic control; H, hypoxia; BM, BM treatment; H+BM, BM treatment under hypoxic conditions.

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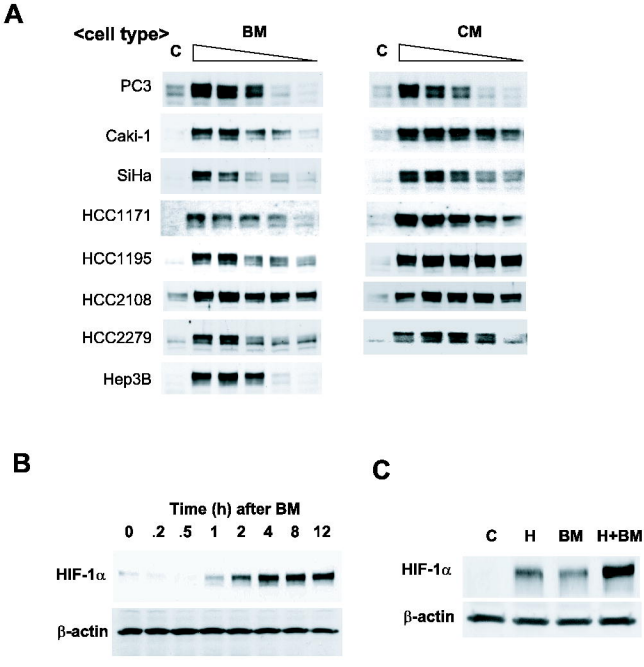
Fig. 5. HIF-1α is required for bafilomycin-induced p21 expression and cell cycle arrest. (A) Bafilomycin-induced HIF-1α expression in MEF cells. Wild-type (HIF-1α+/+) and HIF-1α null (HIF-1α-/-) MEF cells were treated with various concentrations (10, 5, 2, 1, or 0.2 nM) of BM for 4 hours, and then harvested for HIF-1α immunoblotting. (B) Bafilomycin-induced p21 expression. MEF cells were incubated under normoxic or hypoxic conditions for 16 hours, in the absence or in the presence of 10 nM BM, and then harvested for p21 immunoblotting. (C) HIF-1α-dependent cell cycle arrest. After 16 hour incubation, the cell cycle was analyzed in HIF-1α(+/+) (left panel) or HIF-1α(-/-) (right panel) MEF cells. Each bar represents the mean and standard deviation of four experiments. C, normoxic control; H, hypoxia; BM, BM treatment; H+BM, BM treatment under hypoxic conditions. * $p<0.05$ *vs.* G_1-phase population of the C group; # $p<0.05$ *vs.* S-phase population of the C group.

Fig. 6. p21 is required for cell cycle arrest by bafilomycin. (A) Bafilomycin-induced p21 expression in HCT116 cells. p21(+/+) and p21(-/-) HCT116 cells were incubated under normoxic or hypoxic conditions for 16 hours, in the absence or in the presence of 10 nM BM. HIF-1α and p21 levels were analyzed by immunoblotting method. (B) BM-induced cell cycle arrest in HCT116 cells. After 16 hour incubation, the cell cycle was analyzed in p21(+/+) (left panel) or p21(-/-) (right panel) HCT116 cells. Each bar represents the mean and standard deviation of four experiments. C, normoxic control; H, hypoxia; BM, BM treatment; H+BM, BM treatment under hypoxic conditions. * $p<0.05$ *vs.* G_1-phase population of the C group; # $p<0.05$ *vs.* G_2/M-phase population of the C group.

Fig. 7. HIF-1α is required for the anticancer activity of bafilomycin. Nude mice were injected subcutaneously in the flanks with HIF-1α(+/+) or HIF-1α(-/-) MEF cells. After tumors had reached 400 to 500mm^3 in size, the mice were administered an intraperitoneal injection of BM (1 mg/kg) or vehicle (50 μl of DMSO). Tumor sizes were subsequently measured daily for 4 days, and then excised. The growth curves of HIF-1α(+/+) and HIF-1α(-/-) fibrosarcomas are plotted in A-panel and B-panel, respectively. Each data point represents the mean and standard deviation (n = 9 for HIF-1α(+/+) control; n = 9 for HIF-1α(+/+) BM-treated; n = 9 for HIF-1α(-/-) control; n = 7 for HIF-1α(-/-) BM-treated). *

$p<0.05$ *vs.* the vehicle group. Illustrations in the upper panel are tumors excised from mice 4 days after BM injection. (C) Tumor histology. Tumor sections were cut from paraffin blocks and stained with H&E. Cells showing chromatin condensation (arrow) were frequently found in BM-treated HIF-1α(+/+) tumors. Magnification, x200. (D) Detection of apoptotic cells in tumors. Apoptotic cells were detected by TUNEL staining. TUNEL-positive nuclei (arrowed) were frequently found in BM-treated HIF-1α(+/+) tumors. (E) Necrosis in tumors. Massive necrosis was found in BM-treated HIF-1α(+/+) tumors, whereas small, scattered areas of necrosis were present in BM-treated HIF-1α(-/-) tumors. The necrotic areas were evaluated in H&E stained slides and are presented as percentages of total area. Each bar represents the mean and standard deviation of 24 or more specimens. * $p<0.05$ *vs.* the vehicle group. (F) p21 is induced only in BM-treated HIF-1α(+/+) tumors. Tumors were excised the day after BM injection, and quickly frozen in liquid nitrogen. Tumor lysates were assessed by immunoblotting p21, HIF-1α, and β-actin.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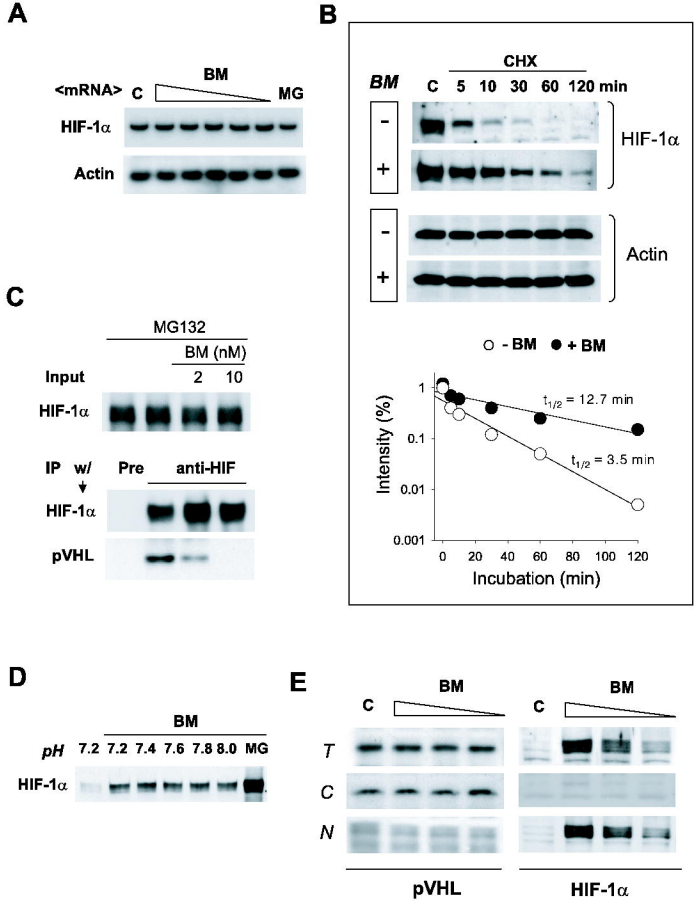
BM
C
CM
C
PC3
Caki-1
SiHa
HCC1171
HCC1195
HCC2108
HCC2279
Hep3B
B
Time (h) after BM
0 .2 .5 1 2 4 8 12
HIF-1α
β-actin
C
C H BM H+BM
HIF-1α
β-actin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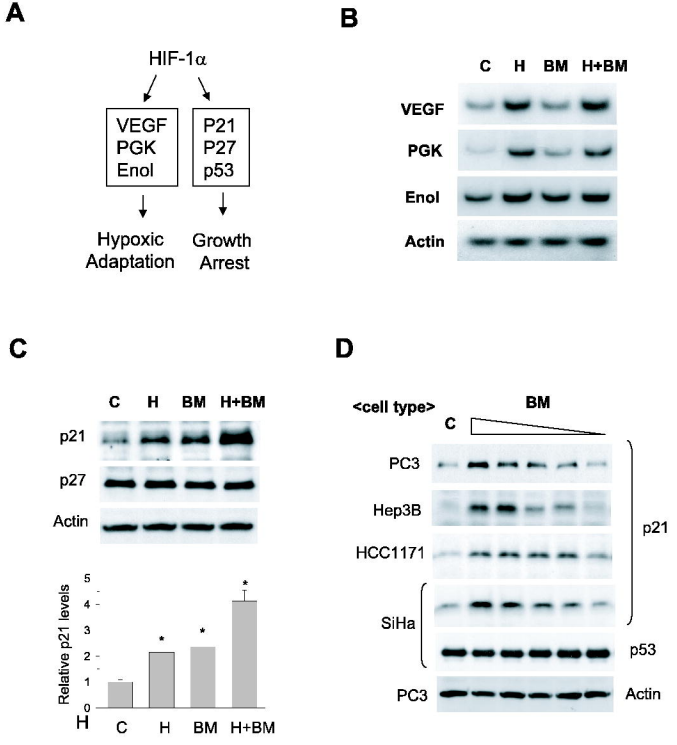
<mRNA>
C
BM
MG
HIF-1α
Actin
B
BM
CHX
C 5 10 30 60 120 min
-
+
HIF-1α
Actin
○ - BM ● + BM
Intensity (%)
1
0.1
0.01
0.001
$t_{1/2}$ = 12.7 min
$t_{1/2}$ = 3.5 min
0 20 40 60 80 100 120
Incubation (min)
C
MG132
BM (nM)
Input
2 10
HIF-1α
IP w/
Pre
anti-HIF
HIF-1α
pVHL
D
BM
pH 7.2 7.2 7.4 7.6 7.8 8.0 MG
HIF-1α
E
C
BM
T
C
N
pVHL
HIF-1α


A
HIF-1α
VEGF
PGK
Enol
P21
P27
p53
Hypoxic Adaptation
Growth Arrest
B
C
H
BM
H+BM
VEGF
PGK
Enol
Actin
C
C
H
BM
H+BM
p21
p27
Actin
Relative p21 levels
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D
<cell type>
BM
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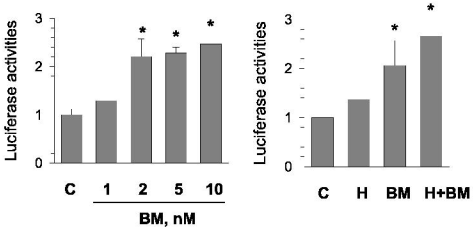
PC3
Hep3B
HCC1171
SiHa
p21
p53
PC3
Actin

E


Luciferase activities
3
2
1
0
*
*
*
C
1
2
5
10
BM, nM
Luciferase activities
3
2
1
0
*
*
C
H
BM
H+BM

F

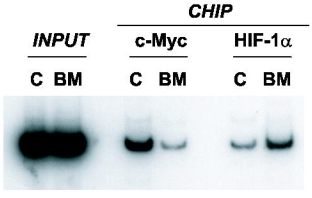
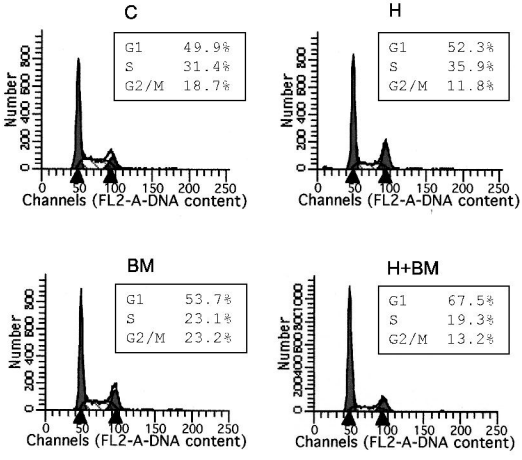
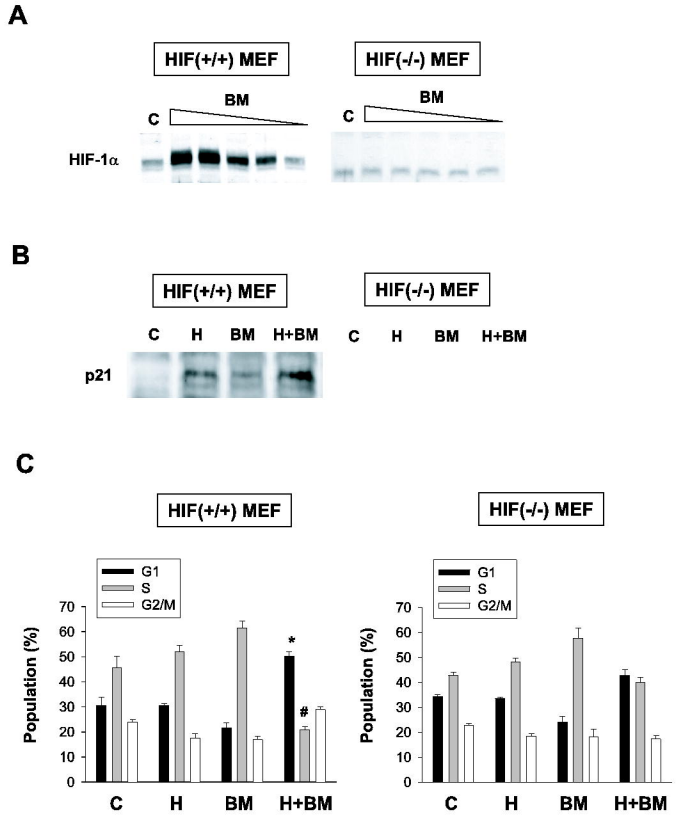
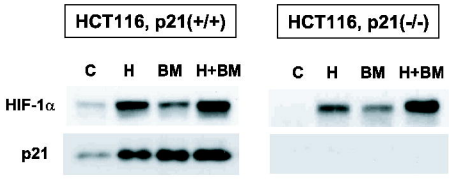

CHIP
INPUT
c-Myc
HIF-1α
C BM
C BM
C BM

Fig. 4

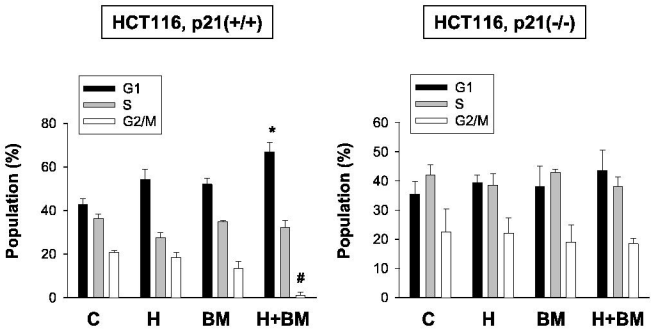

C
G1 49.9%
S 31.4%
G2/M 18.7%
Number
Channels (FL2-A-DNA content)
H
G1 52.3%
S 35.9%
G2/M 11.8%
Number
Channels (FL2-A-DNA content)
BM
G1 53.7%
S 23.1%
G2/M 23.2%
Number
Channels (FL2-A-DNA content)
H+BM
G1 67.5%
S 19.3%
G2/M 13.2%
Number
Channels (FL2-A-DNA content)

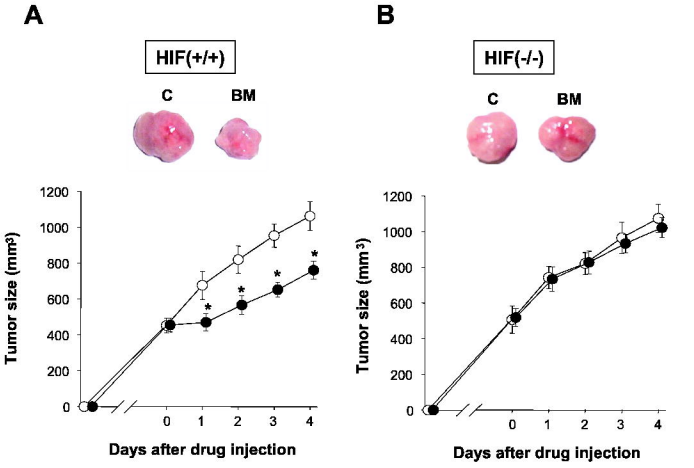

A
HIF(+/+) MEF
HIF(-/-) MEF
C
BM
HIF-1α
B
HIF(+/+) MEF
HIF(-/-) MEF
C H BM H+BM
p21
C
HIF(+/+) MEF
HIF(-/-) MEF
G1
S
G2/M
Population (%)
0 10 20 30 40 50 60 70
*
#
C H BM H+BM

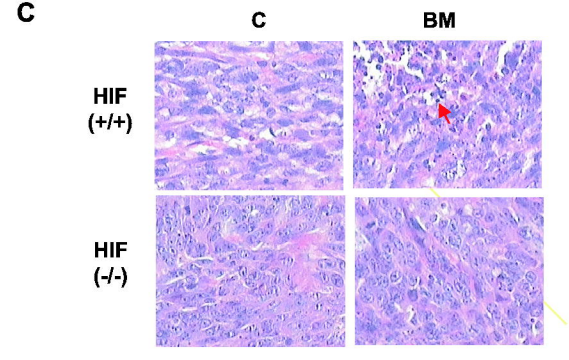
A

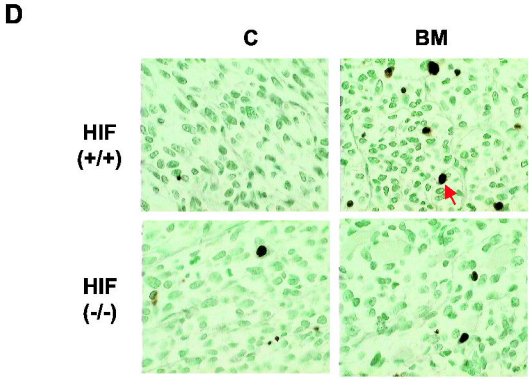

HCT116, p21(+/+)
HCT116, p21(-/-)
C
H
BM
H+BM
C
H
BM
H+BM
HIF-1α
p21

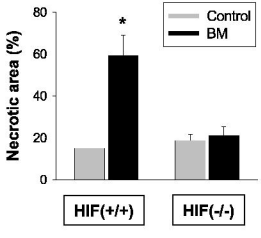
B

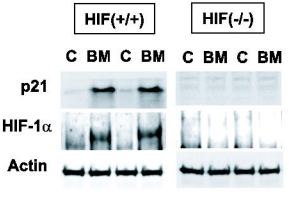

HCT116, p21(+/+)
HCT116, p21(-/-)
G1
S
G2/M
Population (%)
0
20
40
60
80
*
#
C
H
BM
H+BM
G1
S
G2/M
Population (%)
0
10
20
30
40
50
60
C
H
BM
H+BM


A
HIF(+/+)
C
BM
Tumor size (mm³)
1200
1000
800
600
400
200
0
0 1 2 3 4
Days after drug injection
B
HIF(-/-)
C
BM
Tumor size (mm³)
1200
1000
800
600
400
200
0
0 1 2 3 4
Days after drug injection


C
C
BM
HIF
(+/+)
HIF
(-/-)


D
C
BM
HIF
(+/+)
HIF
(-/-)


E
Necrotic area (%)
80
60
40
20
0
*
Control
BM
HIF(+/+)
HIF(-/-)


F
HIF(+/+)
HIF(-/-)
C BM C BM
C BM C BM
p21
HIF-1α
Actin