ARC-111 inhibits hypoxia-mediated hypoxia-inducible factor-1α accumulation

Fanying Meng, Xuan-Thao Nguyen, Xiaohong Cai, Jianxin Duan, Mark Matteucci and Charles P. Hart

ARC-111, a small-molecule topoisomerase I inhibitor, is a potent cytotoxic drug against multiple human cancer cell lines under normoxic conditions (Li *et al.*, *Cancer Res* 2003; 63:8400–8407). In this study, we explore the potential of ARC-111 as a hypoxia-inducible factor-1α inhibitor under hypoxic conditions. The transcription factor, hypoxia-inducible factor-1α, is an essential regulator of tumorigenesis and an attractive molecular target for cancer therapy. We demonstrate that ARC-111 specifically inhibits hypoxia-induced accumulation of hypoxia-inducible factor-1α, but not other short half-life proteins in multiple human cancer cell lines. ARC-111 inhibits hypoxia-inducible factor-1α protein synthesis specifically and does not inhibit protein synthesis globally. We demonstrate that inhibition of hypoxia-inducible factor-1α accumulation by ARC-111 is independent of proteasomal degradation. In addition, we demonstrate using topoisomerase I-resistant cell lines that topoisomerase I is required for ARC-111-mediated hypoxia-inducible factor-1α inhibition. Experiments performed with nocodazole indicate that ARC-111 inhibits hypoxia-inducible factor-1α accumulation in a cell-cycle-independent manner. Analysis of AKT and mammalian target of rapamycin phosphorylation reveals that ARC-111 does not exhibit inhibitory effect on the phosphatidylinositol-3-kinase AKT mammalian target of rapamycin signaling pathway. It has been previously shown that topotecan, a topoisomerase I inhibitor, can also modulate hypoxia-induced hypoxia-inducible factor-1α accumulation (Rapisarda *et al.*, *Cancer Res* 2003; 64:1475–1482). In addition to inhibiting hypoxia-induced accumulation of hypoxia-inducible factor-1α, ARC-111 exhibits antiproliferative effects against multiple human cancer cell lines. We demonstrate that topoisomerase I is required for the antiproliferative effects of ARC-111. Antiproliferative effects of ARC-111, however, are oxygen-independent, which is distinguishable from inhibition of hypoxia-inducible factor-1α accumulation by ARC-111, which is only observed under hypoxia. The results indicate that inhibiting hypoxia-inducible factor-1α accumulation and exhibiting antiproliferation of ARC-111 are through distinct mechanisms of action, which reinforce the potential anticancer effect of ARC-111 on hypoxic tumors.

Anti-Cancer Drugs 2007, **18**:435–445

Keywords: ARC-111, hypoxia-inducible factor-1α, hypoxia, proteasomal degradation and PI3K-AKT-mTOR signaling, topoisomerase I inhibitors

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Received 12 September 2006 **Revised form accepted** 10 November 2006

Introduction

Hypoxia-inducible factor-1α (HIF-1α) plays a pivotal role in tumorigenesis by activating the transcription of genes involved in glycolytic metabolism, cell migration, invasion, angiogenesis, erythropoiesis, cell differentiation and proliferation [1]. HIF-1 is a heterodimeric factor composed of oxygen-sensitive HIF-1α and oxygen-insensitive HIF-1β (also known as the aryl hydrocarbon receptor nuclear translocator) subunits [2]. HIF-1α is the major subunit of HIF-1 and dominates HIF-1 transcriptional activity. HIF-1β is a common subunit for the family of basic helix–loop–helix (bHLH)-Per aryl hydrocarbon receptor nuclear translocator Sim proteins [3]. HIF-1α is constitutively expressed, but rapidly degraded by a ubiquitin-dependent and proteasome-dependent pathway in the presence of oxygen. This degradation occurs through HIF-1α protein hydroxylation by specific HIF-prolyl hydroxylases under normoxia. The hydroxylated protein is then recognized by the von Hippel–Lindau (VHL) tumor suppressor protein [4]. In contrast, in the absence of oxygen, HIF-1α is not hydroxylated, which prevents its interaction with pVHL, and subsequent ubiquitination and degradation. HIF-1β is constitutively expressed and its expression levels are not affected by oxygen concentrations [5]. Following hypoxic stabilization, HIF-1α is translocated to the nucleus and heterodimerizes with HIF-1β, subsequently activating the transcription of genes important for adaptation and survival under hypoxia [6].

HIF-1α is heterogeneously upregulated in common human cancers *in vivo* and their metastases relative to adjacent normal tissue, including breast, prostate, brain, lung and head and neck cancers [7]. Xenografts derived

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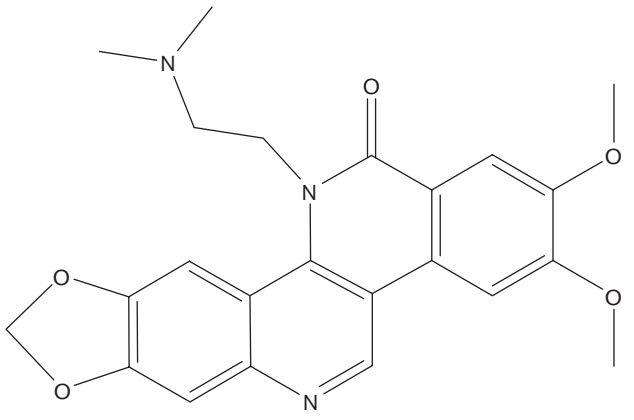
from HIF-1α-deficient hepatoma cells [8] or HIF-1α-deficient Chinese hamster ovary cells [9] are less tumorigenic than parental cell xenografts. HIF-1α knockout mouse embryonic stem (MES) cells also show decreased tumor growth compared with wild-type MES cells [10,11]. Reintroduction of the intact VHL gene into cells derived from renal carcinomas lacking functional VHL restores HIF-1α to normoxic levels [12] and decreases tumorigenesis [13]. In addition, HIF-1α drives transcription of many genes involved in hypoxic tumor cell growth, including vascular endothelial growth factor (VEGF), glucose transporters 1 and 3, glycolytic enzymes, and erythropoietin [14]. HIF-1α is also, however, paradoxically viewed as an inducer of cell cycle arrest and apoptosis as well as an effector of antiproliferation by transactivating antiproliferative and proapoptotic genes [15]. Embryonic stem cells and Chinese hamster ovary cells lacking HIF-1α do not go undergo apoptosis in response to hypoxia [16]. The role of HIF-1α in tumorigenesis is also complicated by the microenvironment of tumors, including pH, reactive oxygen species, ATP and reoxygenation [17,18].

ARC-111 (Topotvale), 8,9-dimethoxy-5-(2-*N*,*N*-dimethylaminoethy)-2,3-methylenedioxy-5-*H*-dibeno[*c*,*h*] [1,6] naphthyridin-6-one (Fig. 1), is a topoisomerase I inhibitor identified and characterized at Rutgers University [19]. ARC-111 exhibited low nanomolar cytotoxicity against a panel of cancer cells [19]. Similarly, ARC-111 was efficacious in mice-bearing human tumor xenografts. ARC-111 was shown to be as active as camptothecin in the HCT-8 colon tumor model [19]. Unlike camptothecin, ARC-111 was not a substrate for the ATP-binding cassette transporter breast cancer-resistant protein and the multidrug resistance pump [19].

Camptothecin class topoisomerase I inhibitors have previously been shown to inhibit hypoxia-induced HIF-1 transcriptional activity and HIF-1α accumulation [20–22]. Topotecan was identified and characterized as a HIF-1α inhibitor using a U251 human glioma cell-based assay [20,21]. Topotecan inhibited HIF-1α by affecting its translation, and topoisomerase I was required for topotecan's action. Concomitant with HIF-1α inhibition, topotecan significantly inhibited U251 human tumor growth *in vivo* with a marked decrease of angiogenesis and expression of HIF-1 target genes in tumor tissue [23].

Fig. 1



Structure of ARC-111.

Here, we describe the biological mechanism by which ACR-111 inhibits hypoxia-mediated HIF-1α accumulation. ARC-111 inhibits hypoxia-mediated accumulation of HIF-1α in a concentration- and time-dependent fashion. Similar to topotecan [21], ARC-111 affects HIF-1α translation rather than its degradation. The inhibition of HIF-1α accumulation by ARC-111 is dependent on topoisomerase I– and independent of proteasome, cell cycle and phosphatidylinositol-3-kinase (PI3K) AKT–mammalian target of rapamycin (mTOR) signaling pathways. In addition, we demonstrate that ARC-111 exhibits antiproliferative effect in topoisomerase-dependent and oxygen-independent manner in multiple human cancer cell lines. The inhibition of HIF-1α by ARC-111 is mechanistically distinguished from its antiproliferative activity.

Materials and methods

Cell lines

Human nonsmall cell lung cancer cell line (H460), human prostate cancer cell line (PC3), human colon cancer cell line (HT29) and human uterus cancer cell line (MESSA) were purchased from the American Type Culture Collection (Manassas, Virginia, USA). Human leukemia cell lines, either sensitive (CCRF-CEM), intermediate resistant (CEM/C1) or highly resistant (CEM/C2) to camptothecin (because of a mutation in topoisomerase I), were also obtained from the American Type Culture Collection. Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum, penicillin/streptomycin and 2 mmol/l glutamine. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2. Hypoxia treatment was performed by placing cells in a hypoxic environment equilibrated with 0.1% O_2, 5% CO_2 and 94.9% nitrogen for 4 h.

Reagents and antibodies

ARC-111 was synthesized at Threshold Pharmaceuticals, (Redwood City, California, USA), MG132, camptothecin, nocodazole and rapamycin were purchased from Sigma Aldrich (St Louis, Missouri, USA). [^{14}C]Leucine was purchased from GE Healthcare (Piscataway, New Jersey, USA). AlamarBlue, AKT antibody and phosphorylated mTOR were obtained from BioSource International (Camarillo, California, USA). Topotecan was obtained from TopoGen (Port Orange, Florida, USA). LY294002 was obtained from EMD Biosciences (San Diego,

California, USA). Monoclonal anti-HIF-1α antibody was obtained from BD Transduction Laboratories (San Diego, California, USA). Monoclonal anti-HIF-1β antibody was obtained from Novus Biologicals (Littleton, Colorado, USA). NFκB antibody was obtained from Upstate Biotechnology (Lake Placid, New York, USA). Antibodies against IκBα and phosphorylated AKT were obtained from Cell Signaling Technology (Danvers, Massachusetts, USA). Actin antibody was obtained from Chemicon International (Temecula, California, USA).

Preparation of nuclear extract preparation and immunoblotting (Western blot)

Cells were plated at a density of 7×10^5 for H460 and PC3 cells, and 1.4×10^6 cells for leukemia cell lines (CCRF-CEM, CEM/C1 and CEM/C2) into a 60-mm glass dish, and then maintained in a 37°C incubator (5% CO_2) for 2 days. Cells were pretreated with compounds at different concentrations for 4 h under normoxic condition at 37°C and then continuously treated for an additional 4 h under either normoxia (21% O_2) or hypoxia (0.1% O_2). At the end of this incubation, the dishes were placed on ice and then the cells were washed rapidly twice with cold phosphate-buffered saline (4°C). For nuclear extracts, cells were lysed with buffer A (10 mmol/l Tris, pH 7.5; 1.5 mmol/l $MgCl_2$; 10 mmol/l KCl and protease inhibitor) and buffer C (0.5 mol/l NaCl; 20 mmol/l Tris pH 7.5; 1.5 mmol/l $MgCl_2$, 20% glycerol and protease inhibitors), sequentially. For whole-cell extracts, cells were lysed in lysis buffer [150 mmol/l NaCl, 10 mmol/l Tris (pH 7.0), 10 mmol/l EDTA, 1% Triton X-100, 0.5% deoxycholate, 1 mmol/l Na_2VO_4, 1 mmol/l PMSF and 1 μg/ml each of leupeptine/pepstatin A/aprotinin). The protein concentration was measured using either Bio-Rad protein assay (Hercules, California, USA) or BCA assay (Sigma Aldrich). Equal amount of protein was loaded in each lane of an sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (Invitrogen, Carlsbad, California, USA). After electrophoresis and electrotransfer to polyvinylidine diflouride membrane, the membrane was blocked with TBST (10 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl and 0.1% Tween-20) containing 5% nonfat dry milk overnight at 4°C, incubated with primary antibodies against HIF-1α, HIF-1β, AKT, phosphorylated AKT, phosphorylated mTOR or actin for 2 h or overnight for antibodies against IκB and NFκB at 4°C, and washed with TBST. The specific binding of primary antibodies was detected with the enhanced chemiluminescence reagent using horseradish peroxidase-conjugated secondary antibody. The intensity of the protein bands was quantified using National Institutes of Health Image software (Scion Corporation Frederick, Maryland).

De-novo protein synthesis

Cells were seeded at a density of 3×10^5 cells per well in six-well plates in media containing 10% fetal bovine serum and antibiotics. After 24 h for attachment, cells were washed twice with leucine-free medium and exposed to leucine-free media containing 10% dialyzed serum for 2.5 h in the absence or presence of compounds at a desired concentration at 37°C. Cells were then labeled by incubation with 1.25 μCi of L-[U-^{14}C]leucine for 90 min in 37°C incubator. After separation of incorporated from free [U-^{14}C]leucine [24], the incorporated [U-^{14}C]leucine was determined using a scintillation counter.

Hypoxia-inducible factor-1α protein translation assay

Cells were exposed to ARC-111 in regular medium for 5 h, and then methionine- and cysteine- free media for 2 h in the presence or absence of ARC-111. Cells were then labeled by incubation with methionine- and cysteine-free medium containing 1.25 μCi of [^{35}S]methionine and [^{35}S]cysteine for the indicated times. Total cell lysates were subjected to immunoprecipitation using anti-HIF-1α monoclonal antibody, and subsequently Protein G-agarose beads were added and incubation continued for an additional 1 h. After centrifugation and wash, immunoprecipitation products were separated on a 10% sodium dodecyl sulfate gel. The gel was dried and autoradiographed.

Cell cycle analysis

H460 cells were seeded at a density of 1.5×10^6 cells per well in a six-well plate. After 24 h for attachment, ARC-111 at desired concentrations was added and incubated for 24 h. Cells were trypsinized, centrifuged and fixed in 75% ethanol for at least 24 h at −20°C. Cell cycle distribution was determined using Guava Cell Cycle reagent and flow cytometry (Guava, Hayward, California, USA).

In-vitro proliferation assay

For cell proliferation under hypoxia, cells were seeded at a density ranging from 20 000 to 30 000 cells onto 24-well glass insert wells. For proliferation under normoxia, cells were seeded at a density ranging from 4000 to 6000 cells per well of 96-well plate. Cells were incubated at 37°C in 5% CO_2, 95% air and 100% relative humidity for 24 h before addition of compounds. The cell population for each cell line at the time of drug addition (T_0) was measured using the AlamarBlue assay. Compounds were solubilized in 100% dimethylsulfoxide at 200 times the desired final test concentration. Following drug addition, the plates were incubated for an additional 72 h at 37°C, 5% CO_2 and 100% relative humidity under normoxia or hypoxia. At the end of incubation, viable cells were quantified using the AlamarBlue assay. Growth inhibition of 50% (IC_{50}) was calculated using Prism software (Prism software Corporation, Irvine, California, USA) at each of the drug concentrations.

Results

ARC-111 specifically inhibits the accumulation of hypoxia-inducible factor-1α in a concentration and time-dependent manner

The effect of ARC-111 (Fig. 1) on hypoxia-induced accumulation of HIF-1α was examined. After exposure to 0.1% O_2 for 4 h, HIF-1α accumulation in PC3 (Fig. 2a) and H460 cells (Fig. 2b) became apparent. In the presence of ARC-111, hypoxia-induced HIF-1α accumulation was inhibited in a concentration-dependent manner, and reached complete inhibition at 100 nmol/l in both H460 cells and PC3 cells. Camptothecin, a known topoisomerase I and HIF-1α inhibitor, was used as a positive control in this study. In contrast, actin, NF-κB, and IκBα were not affected by treatment with ARC-111 in PC3 and H460 cells. HIF-1β was not affected by ARC-111 treatment at 100 nmol/l in PC3 cells, but was slightly inhibited at 400 nmol/l. HIF-1β expression in H460 cells was, however, modulated quite differently. In H460 cells, expression of HIF-1β was oxygen-dependent and inhibited by ARC-111 in a concentration-dependent fashion, which matched the effect seen on HIF-1α expression. The HIF-1β in the nuclear fraction of H460 cells likely represented HIF-1α/HIF-1β complexes. A similar observation has been reported in Hepa-1 and H4 cells [25].

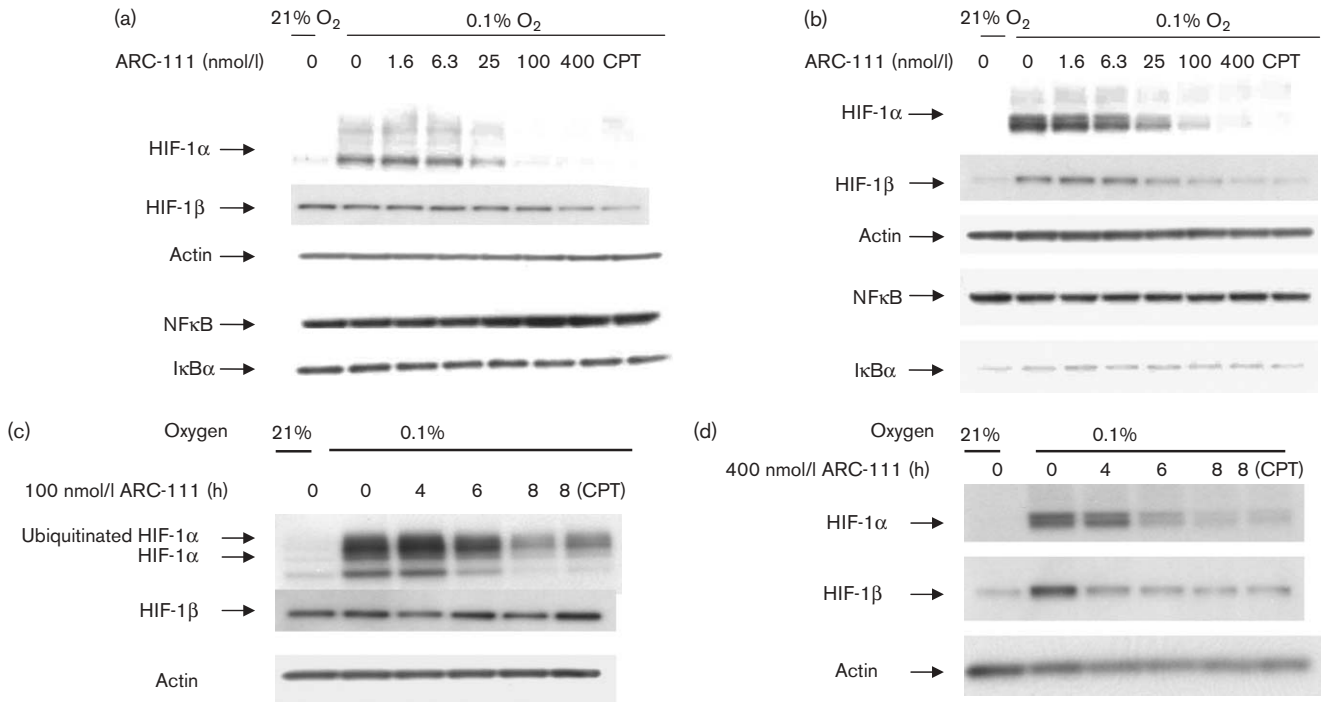
A time-course experiment investigated the kinetics of ARC-111-mediated inhibition of HIF-1α accumulation (Fig. 2c and d). The initial inhibition of HIF-1α accumulation was detected at 6 h, and greater inhibition was observed after longer treatment in both PC3 cells and H460 cells. Similar to the results shown in Fig. 2a and b, the protein level of HIF-1β was constant in PC3 cells, but resembled HIF-1α expression in H460 cells, in which it decreased. Equal loading was confirmed by actin immunoblotting.

These results demonstrate that ARC-111 inhibits hypoxic induction of HIF-1α accumulation in a concentration and time-dependent manner.

ARC-111 inhibits hypoxia-induced accumulation of hypoxia-inducible factor-1α in a proteasomal-independent manner

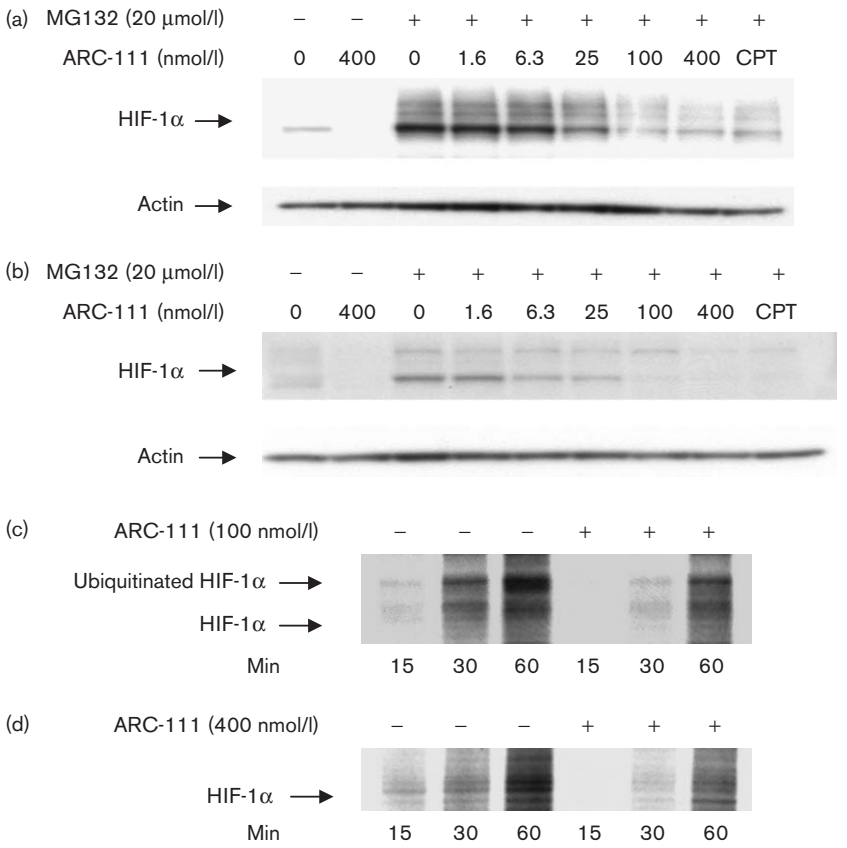
Under normoxic conditions, HIF-1α protein is degraded rapidly and continuously by ubiquitination and the proteasomal system upon hydroxylation of Pro-402 and Pro-564 [3,26]. In contrast, under hypoxic conditions, HIF-1α protein is stabilized and escapes proteasomal degradation. To investigate whether inhibition of HIF-1α accumulation by ARC-111 is mediated by the proteasomal system, we performed an experiment in the

Fig. 2



ARC-111 specifically inhibits hypoxia-induced accumulation of hypoxia-inducible factor-1α (HIF-1α) protein in a concentration- and time-dependent manner. (a) PC3 cells or (b) H460 cells were pretreated with ARC-111 at the indicated concentrations under normoxia for 4 h and then continuously treated with ARC-111 for additional 4 h under hypoxic conditions. PC3 cells (c) or H460 cells (d) were treated with either 100 or 400 nmol/l of ARC-111 at the indicated time-points under normoxic or hypoxic conditions. The nuclear extracts were prepared, and immunoblotted with antibodies against HIF-1α and HIF-1β. Equal amount of loading were confirmed by immunoblotting with antibody against actin. The cellular proteins from whole cell lysates were immunoblotted with antibodies against NFκB and IκBα. Camptothecin (CPT) was used as a positive control.

Fig. 3



ARC-111 inhibits hypoxia-induced accumulation of hypoxia-inducible factor-1α (HIF-1α) in a proteasomal-independent manner and decreases HIF-1α protein translation. PC3 cells (a) or H460 cells (b) were treated for 8 h under normoxic conditions in the presence or absence of proteasome inhibitor, MG-132. The nuclear extracts were immunoblotted with antibody against HIF-1α and actin. PC3 cells (c) and H460 cells (d) were treated with ARC-111 at the indicated concentration for 5 h in normoxia. Cells were then starved in methionine- and cysteine-free media for 2 h in the presence of ARC-111. Total extracts were harvested at the indicated times after the addition of ^{35}S-labeled methionine and cysteine for the indicated times, and then immunoprecipitated with HIF-1α antibody. The immunoprecipitated product was separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gel was dried and autoradiographed.

presence of the proteasomal inhibitor MG-132. As shown in Fig. 3a and b, and as compared with a nontreated control sample, MG132-induced nuclear accumulation of HIF-1α protein in the presence of oxygen. ARC-111 inhibited MG132-mediated HIF-1α accumulation in a concentration-dependent manner in both PC3 cells and H460 cells. By comparison of Figs 2 and 3, a similar potency can be observed for ARC-111-inhibitable HIF-1α accumulation induced either by hypoxia or by the proteasomal inhibitor MG132 in the presence of oxygen.

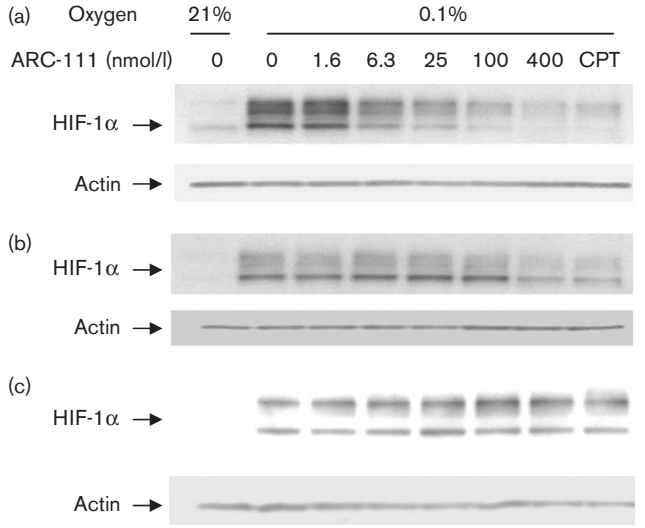
This result indicates that ARC-111-inhibited HIF-1α accumulation is not because of destabilization of HIF-1α protein via the proteasomal system.

ARC-111 decreases hypoxia-inducible factor-1α protein translation

To address whether ARC-111-mediated inhibition of HIF-1α was related to decreased HIF-1α protein translation, we performed metabolic labeling experiments under normoxic conditions. As shown in Fig. 3c and d, ^{35}S-labeled HIF-1α progressively accumulated in the absence of ARC-111. After 30 min, the newly synthesized HIF-1α became visible in vehicle-treated samples, but not in the presence of ARC-111 in both PC3 (Fig. 3c) and H460 cells (Fig. 3d). After 60 min of labeling, newly synthesized HIF-1α was detectable in the presence of ARC-111, but at much lower levels than that in vehicle-treated samples.

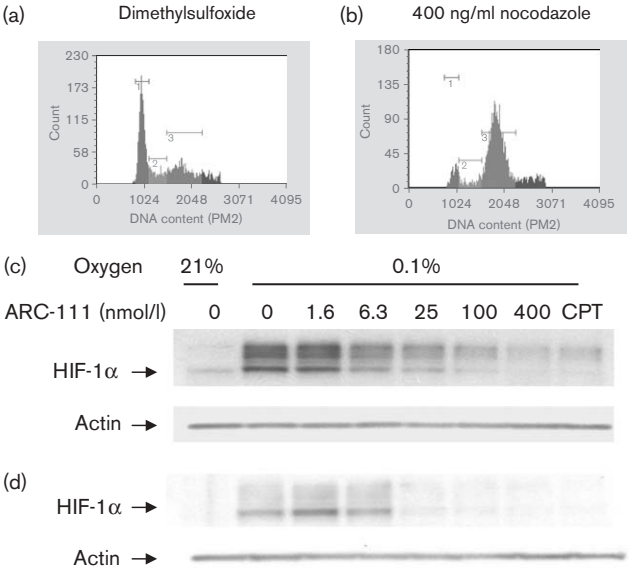
To assess the specificity of ARC-111-mediated HIF-1α inhibition at the translational level, we evaluated the effect of ARC-111 on protein synthesis generally. Under the same experimental conditions as described in Fig. 3, ARC-111 did not significantly inhibit global protein synthesis, that was monitored by incorporation of [^{14}C]leucine (data not shown). In contrast, a known protein synthesis inhibitor, cycloheximide, achieved greater than 90% inhibition under the same experimental conditions (data not shown).

Fig. 4



Topoisomerase I is required for ARC-111-mediated inhibition of hypoxia-induced accumulation of hypoxia-inducible factor-1α (HIF-1α). (a) Sensitive CCRF-CEM, (b) moderate resistant CEM/C1 and (c) highly resistant CEM/C2 cell lines were treated with ARC-111 at the indicated concentrations under normoxia for 4 h, and then continuously treated for additional 4 h under hypoxic conditions. The nuclear extracts were immunoblotted with antibody against HIF-1α and actin.

Fig. 5



Inhibition of hypoxia-induced accumulation of hypoxia-inducible factor-1α (HIF-1α) by ARC-111 is cell-cycle-independent. CCRF cells were pretreated with either vehicle (a) or synchronized with 400 ng/ml of nocodazole (b) overnight, and then the cell cycle distribution was monitored using Guava Cell Cycle reagent and flow cytometry. Cells were treated with ARC-111 at the indicated concentrations for 4 h under normoxia either without nocodazole pretreatment (c) or with nocodazole pretreatment (d). Before harvest, cells were continuously treated with ARC-111 for 4 h at the indicated concentrations under hypoxic conditions. The nuclear extracts were prepared and immunoblotted with antibodies against HIF-1α and actin.

This result suggests that ARC-111 specifically inhibits HIF-1α protein translation, but not protein synthesis generally.

Topoisomerase I is required for ARC-111-mediated inhibition of hypoxia-induced accumulation of hypoxia-inducible factor-1α

To investigate whether topoisomerase I was required for the inhibition of HIF-1α by ARC-111, we tested the effect of ARC-111 on hypoxia-induced HIF-1α accumulation in human leukemia cell lines, including both camptothecin-sensitive (CCRF-CEM) and camptothecin-resistant (CEM/C1 and CEM/C2) cell lines. Of the two resistant cell lines chosen, one is moderately resistant (CEM/C1) and the other is highly resistant (CEM/C2) because of a mutation in Top 1. As expected, ARC-111 decreased hypoxia-induced HIF-1α accumulation in the camptothecin-sensitive cell line (CCRF-CEM, Fig. 4a) and showed a similar potency in other human cancer cell lines (Fig. 2). Whereas ARC-111 showed inhibitory effects only at 400 nmol/l in the CEM/C1 moderately resistant cell line (Fig. 4b), addition of ARC-111 did not affect HIF-1α accumulation in the CEM/C2 highly camptothecin-resistant cell line at all concentrations tested (Fig. 4c).

These results demonstrate a correlation between sensitivity of HIF-1α inhibition by ARC-111 and topoisomerase I resistance, and support the hypothesis that ARC-111 acts on HIF-1α by targeting topoisomerase 1.

Table 1 Quantification of cell cycle distribution

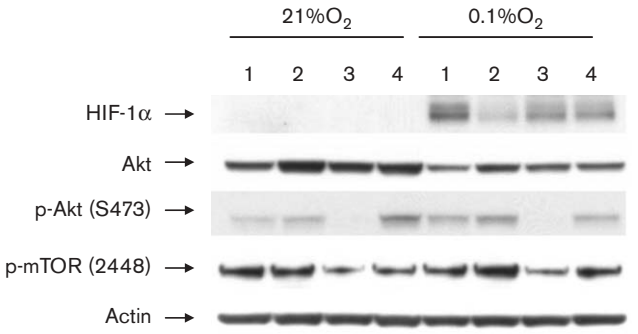
Treatment	% G_0/G_1	% S	% G_2/M
Dimethylsulfoxide	48	14	29
Nocodazole	9	8	69

Inhibition of hypoxia-induced accumulation of hypoxia-inducible factor-1α by ARC-111 is cell cycle-independent

To evaluate whether ARC-111-mediated inhibition of HIF-1α accumulation was dependent on cell cycle, we synchronized cells at the G_2/M phase before ARC-111 treatment. As shown in Fig. 5a, vehicle-treated cells demonstrated a normal cell cycle distribution with 48% at G_0/G_1, 14% at S and 29% at G_2/M (Table 1). Nocodazole (Fig. 5b) arrested the majority of the cell population at the G_2/M phase with 69% cells at G_2/M phase, 9% at G_0/G_1 and 8% at S phase (Table 1). Interestingly, addition of ARC-111 to the arrested cells resulted in a concentration-dependent inhibition of HIF-1α accumulation (Fig. 5d). The inhibitory concentration in cell cycle-arrested cells was similar with that observed in untreated cells (Fig. 5c).

This result supports the conclusion that ARC-111-mediated inhibition of hypoxia-mediated HIF-1α accumulation is independent of cell cycle.

Fig. 6

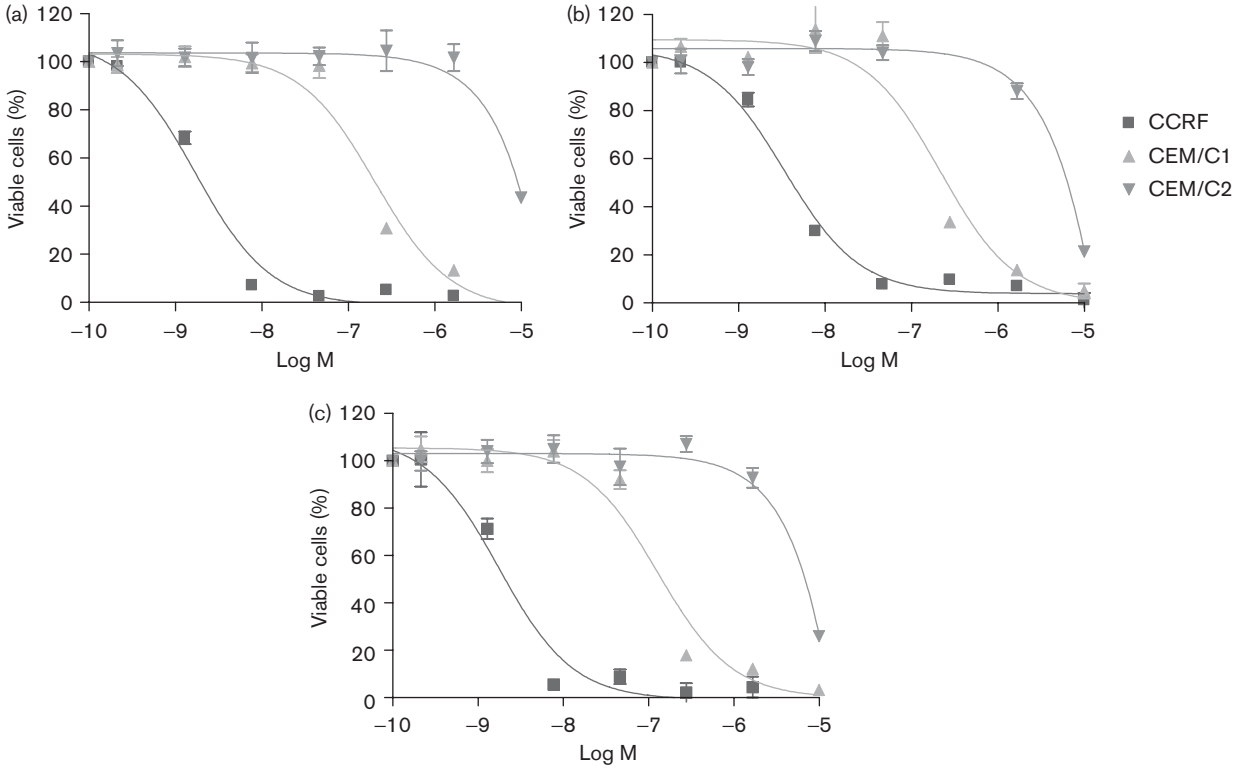


Inhibition of hypoxia-induced accumulation of HIF-1α by ARC-111 is independent of phosphatidylinositol-3-kinase–AKT–mammalian target of rapamycin (mTOR) signaling pathway. H460 cells were pretreated for 4 h under normoxia, and continuously treated for additional 4 h under either normoxia or hypoxia in the presence of dimethylsulfoxide vehicle (1), 100 nmol/l ARC-111 (2), 20 μmol/l LY294002 (3) or 200 nmol/l rapamycin (4). The expressional level of proteins was detected using specific antibodies as indicated.

Inhibition of hypoxia-inducible factor-1α accumulation by ARC-111 is phosphatidylinositol-3-kinase–AKT–mammalian target of rapamycin signaling pathway-independent

PI3 kinase signaling has been linked to HIF-1α regulation. To assess whether the ARC111 effect on HIF-1α inhibition was mediated by the PI3K signaling pathway, cells were treated with ARC-111, the PI3k inhibitor LY294002 and the mTOR inhibitor rapamycin, and then the expression levels of HIF-1α, total AKT, phosphorylated AKT and phosphorylated mTOR under normoxia and hypoxia were measured. H460 cells express AKT, phosphorylated AKT and phosphorylated mTOR constitutively. As shown in Fig. 6, ARC-111 inhibited hypoxia-mediated HIF-1α accumulation without affecting the expression of total AKT, phosphorylated AKT and mTOR under both normoxic and hypoxic conditions. In contrast, the PI3K inhibitor LY294002 inhibited hypoxia-induced HIF-1α accumulation and phosphorylation of AKT and mTOR under both normoxia and hypoxia without affecting total AKT expression. The mTOR inhibitor rapamycin inhibited hypoxia-induced HIF-1α accumulation and phosphorylation of mTOR without affecting total AKT and phosphorylated AKT.

Fig. 7

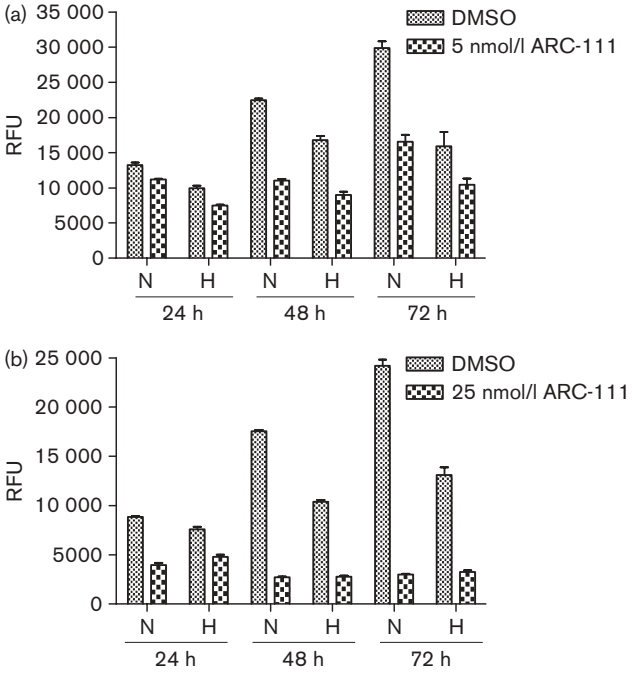


Topoisomerase I is required for ARC-111-mediated antiproliferation. Sensitive (CCRF-CEM) and resistant (CEM/C1 and CEM/C2) human leukemia cell lines were treated with camptothecin (a) topotecan (b) and ARC-111 (c). After 3 days of treatment, viable cells were detected using an AlamarBlue assay.

Table 2 Antiproliferative effect of ARC-111 against multiple cancer cell lines under normoxia and hypoxia

Tumor cell line	Tumor type	IC_{50}(nmol/l)	
		Normoxia	Hypoxia
H460	Non-small cell lung	3.8 ± 0.3	4.4 ± 1.1
PC3	Prostate	8.7 ± 3.3	12 ± 2.8
HT29	Colon	31 ± 1.4	28 ± 2.8
MESSA	Uterus	6.6 ± 3.8	6.8 ± 2.4

Fig. 8



HIF-1α inhibition by ARC-111 is distinguishable from antiproliferative effects of ARC-111. H460 cells were seeded and cultured for 3 days under either normoxia (N) or hypoxia (H) in the presence of ARC-111 at either 5 (a) or 25 (b) nmol/l The viable cells were quantified using the AlamarBlue assay. DMSO, dimethylsulfoxide.

This result demonstrates that inhibition of hypoxia-inducible HIF-1α accumulation by ARC-111 is independent of the PI3K–AKT–mTOR signaling pathway, even though PI3K–AKT–mTOR signal transduction is involved in the regulation of HIF-1α.

Topoisomerase I is required for antiproliferative effects of ARC-111

To address whether topoisomerase I was required for ARC-111 cytotoxicity, two topoisomerase mutant cell lines, CEM/C1, a moderately resistant cell line, and CEM/C2, a highly resistant cell line, were used. We first confirmed the sensitivity of the three cell lines to camptothecin. As shown in Fig. 7a, the resistance profiles of three cell lines were as expected with IC_{50} values ranging from low nanomolar (sensitive line; CCRF-CEM) to micromolar (highly resistant cell line; CEM/C2). Similar to camptothecin, topotecan, a camptothecin analog, showed a similar resistance profile (Fig. 7b). When cells were treated with ARC-111 at different concentrations, the order of sensitivity to ARC-111 was the same as that to camptothecin and topotecan (Fig. 7c). The data demonstrated that cytotoxic potency positively correlated with the degree of topoisomerase resistance.

These results show that the potency of ARC-111-mediated antiproliferation is topoisomerase I-dependent.

Antiproliferative effect of ARC-111 is independent of oxygen level

To investigate whether the antiproliferative activity of ARC-111 was dependent on oxygen level, we performed *in-vitro* proliferation assays with a panel of human cancer cell lines under both normoxia and hypoxia. As shown in Table 2, ARC-111 was a potent antiproliferative drug with IC_{50} values in a low nanomolar range against four human cancer cell lines after treatment with ARC-111 for 3 days. By direct comparison, similar IC_{50} values from *in-vitro* proliferation assays were obtained when cells were cultured under either normoxia or hypoxia (Table 2). Unlike HIF-1α inhibition by ARC-111, which was only observed under hypoxic condition owing to the rapid degradation of HIF-1α in the presence of oxygen, ARC-111-mediated antiproliferation is independent of oxygen level.

To investigate the role of HIF-1α in ARC-111-mediated antiproliferation under hypoxia, we performed a more detailed *in-vitro* proliferation assay under hypoxia with two concentrations of ARC-111, 5 and 25 nmol/l. As shown in Fig. 8a and b, cells proliferated at much slower rate under hypoxia compared with that under normoxia. Under hypoxia, 5 nmol/l ARC-111 (Fig. 8a) was able to inhibit cell proliferation without inhibiting HIF-1α (cf. with Fig. 2). This observation further confirmed that inhibiting HIF-1α and antiproliferation are two distinct actions of ARC-111. In addition, we demonstrated that 25 nmol/l of ARC-111 inhibited both cell proliferation (Fig. 8b) and HIF-1α accumulation under hypoxia (Fig. 2).

Discussion

In this study, we show that ARC-111 inhibits hypoxia-induced HIF-1α accumulation in a dose-dependent fashion in multiple human cancer cell lines. Inhibition of HIF-1α by ARC-111 is through specific inhibition of HIF-1α at the translational level. ARC-111 does not significantly interfere with global protein synthesis. Furthermore, the inhibitory effect of ARC-111 on HIF-1α is dependent on topoisomerase activity, but independent of proteasomal degradation, cell cycle and the PI3K–AKT–mTOR signaling pathway. In addition, we

demonstrate that ARC-111 is a potent antiproliferative reagent in a spectrum of human cancer cell lines, including prostate, lung, colon, uterus and leukemia cancer cell lines. The antiproliferative activity of ARC-111 is hypoxia-independent and mechanistically distinguishable from HIF-1α inhibition.

HIF-1α is regulated by either protein translation or proteasome-dependent degradation. In general, upregulation of HIF-1α in tumor cells is mediated by enhanced HIF-1α protein synthesis via a variety of growth factors, whereas hypoxia-induced HIF-1α accumulation is because of the shutdown of proteasomal degradation system in the absence of oxygen. We found that ARC-111 specifically decreased the rate of HIF-1α translation. Unlike cycloheximide, a known protein synthesis inhibitor, ARC-111 did not significantly inhibit global protein synthesis. In the presence of the proteasome inhibitor MG132, ARC-111 showed concentration-dependent inhibition of HIF-1α accumulation. Similar to ARC-111, topotecan has also been shown to inhibit hypoxia-mediated HIF-1α accumulation by decreasing HIF-1α translation [21]. In contrast, geldanamycin decreased hypoxia-induced HIF-1α accumulation via the enhancement of HIF-1α degradation via proteasome pathway [27].

Similar to camptothecin, ARC-111 induced topoisomerase I-mediated DNA breaks [19]. These breaks were shown to be reversible on a brief heat treatment, suggesting that ARC-111 traps reversible topoisomerase I-cleavable complexes. ARC-111 gives rise to a different cleavage pattern than camptothecin, suggesting that the cleavage specificity of these two drugs is not the same. topoisomerase I is required for ARC-111-mediated cytotoxicity. Cells resistant to camptothecin and topotecan, because of a genetic mutation in the topoisomerase I gene were not sensitive to ARC-111-related cytotoxicity. A critical question is whether topoisomerase I inhibition is required for the inhibition of HIF-1α protein accumulation by ARC-111. Our evidence shows that topoisomerase I is the biochemical target linking the activity of ARC-111 to the inhibition of HIF-1α accumulation, as has been shown for topotecan [20,21]. Cells resistant to ARC-111-mediated cytotoxicity owing mutations in topoisomerase I are no longer sensitive to the inhibition of HIF-1α accumulation by ARC-111.

ARC-111 specifically inhibits hypoxia-mediated accumulation of HIF-1α. We have provided two lines of evidence to support this observation. The first line of evidence is that inhibition of HIF-1α accumulation by ARC-111 is not because of inhibition of protein synthesis generally. Our [^{14}C]leucine labeling experiment shows that ARC-111 does not affect general protein synthesis to the same extent it inhibits HIF-1α protein synthesis. The second line of evidence is that ARC-111 does not inhibit other proteins with a short half-life, such as NFκB and IκBα.

Camptothecin and topotecan are S-phase-specific targeting agents that require ongoing DNA replication to exert their cytotoxic activity. Conversely, topotecan-mediated inhibition of hypoxia-induced HIF-1α accumulation is independent of cell cycle [21]. Similar to that obtained for topotecan, our data show that the ability of ARC-111 to decrease hypoxia-mediated HIF-1α accumulation is also cell cycle-independent, because the inhibitory effect remains when cells are treated with the microtubule depolymerizing agent nocodazole, which arrests cells at the G_2/M phase.

HIF-1 is a key factor in cancer progression [8,22]. Despite accumulating evidence suggesting a correlation between HIF-1 overexpression and poor prognosis in human cancers, HIF-1α is still controversial as a target for cancer therapy [8,10,16,28]. Currently, different approaches are being used to inhibit HIF-1 activity, including small-molecule HIF-1α inhibitors, such as topotecan, YC-1, 2ME2 and geldenamycin, PI3K–AKT–mTOR inhibitors [29,30], MEKK inhibitors [31] and thioredoxin reductase inhibitors [32]. Antisense oligonucleotides against HIF-1α [33], dominant-negative forms of HIF-1α [34], and a peptide that inhibits the binding between HIF-1α and the coactivator p300/CBP are also under investigation [35].

AKT/PI3K signaling has been implicated in the induction of HIF-1α accumulation and VEGF gene expression. The hypoxia-inducible HIF-1α accumulation can be blocked by inhibitors of PI3K and FRAP, such as LY294002 and rapamycin, respectively [30]. HIF-1-dependent gene transcription is blocked by dominant-negative AKT or PI3K and by wild-type PTEN, whereas transcription is stimulated by constitutively active AKT or dominant-negative PTEN [30]. LY294002 and rapamycin also inhibit growth factor and mitogen-induced secretion of VEGF, the product of a known HIF-1 target gene, thus linking the PI3K/PTEN/AKT/FRAP pathway, HIF-1 and tumor angiogenesis [36]. Most recent evidence suggests a possible role of AKT in the degradation of HIF-1α [29]. PTEN regulates hypoxia and insulin-like growth factor-1-induced angiogenic gene expression by regulating AKT activation of HIF-1 activity [37]. It has been reported that PI3K–AKT survival signaling pathway plays an important role in topotecan-mediated cytotoxicity [38]. Topotecan treatment promoted AKT dephosphorylation that led to the inactivation of AKT in human lung cancer A549 cells. Transfection of the constitutively active *AKT* cDNA into A549 cells resulted in the reduction of the cytotoxic effect of topotecan. In addition, VEGF- and basic fibroblast growth factor-induced vascular endothelial cell migration by topotecan has been linked to

downregulation of the PI3K–AKT signaling pathway [39]. Inhibition of HIF-1α protein translation by topotecan, however, is independent of the PI3K–AKT–mTOR signaling pathway in U251 cells, although the PI3K–AKT–mTOR pathway is indeed involved in hypoxia induction of HIF-1α accumulation [21].

In this study, the effect of ARC-111 on AKT signaling was examined. Our data demonstrate that ARC-111 at a concentration capable of inhibiting hypoxia-mediated HIF-1α accumulation did not have any effect on the phosphorylation of AKT and mTOR, supporting the hypothesis that ARC-111 action on HIF-1α is independent of the PI3K–AKT–mTOR signaling pathway.

Although HIF-1α plays an essential role in tumor growth and progression by upregulating genes necessary for angiogenesis, cell proliferation and the hypoxic adaptive response, it is also paradoxically viewed as an inducer of cell cycle arrest and apoptosis and an effector of antiproliferation by transactivating antiproliferative and proapoptotic genes. This makes it unclear whether HIF-1α inhibitors will provide a selective advantage or disadvantage to tumor progression under hypoxic conditions. YC-1 was reported as a HIF-1α inhibitor by reducing the protein level of HIF-1α and inhibiting the expression of hypoxia-inducible genes in cultured hepatoma cells [40]. More recently, a new distinct biological activity to arrest cell cycle and to induce apoptosis has been described for YC-1 [41]. The combination of HIF-1α inhibition and induction of apoptosis is likely to contribute to the anticancer effect of YC-1. In this study, we demonstrated that ARC-111 inhibited both HIF-1α and proliferation under hypoxia. Furthermore, our data imply that the inhibition of HIF-1α accumulation and induction of cytotoxicity under hypoxia by ARC-111 are through distinct mechanisms of action. ARC-111 inhibited cell proliferation under both normoxia and hypoxia in multiple human cancer cell lines. In contrast, inhibition of HIF-1α accumulation by ARC-111 was only observed under hypoxia because HIF-1α undergoes rapid degradation in the presence of oxygen. The two distinguishable biological actions of ARC-111 reinforce its potential anticancer effects on hypoxic tumors.

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