

9- β -D-Arabinofuranosyl-2-fluoroadenine Inhibits Expression of Vascular Endothelial Growth Factor through Hypoxia-Inducible Factor-1 in Human Ovarian Cancer Cells

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ABSTRACT

Ovarian cancer is the leading cause of death from gynecological malignancy and has the worst prognosis of all gynecological cancers. Vascular endothelial growth factor (VEGF) plays an important role in ovarian cancer development. 9- β -D-Arabinofuranosyl-2-fluoroadenine (Fara-A), a nucleotide analog, is frequently used in treating certain types of cancer. However, the effectiveness of Fara-A on ovarian cancer cells is unknown. In this study, we found that Fara-A inhibited VEGF expression in human ovarian cancer cells. Fara-A inhibited VEGF transcriptional activation through hypoxia-inducible factor 1 (HIF-1). HIF-1 is composed of HIF-1 α and -1 β subunits. Fara-A inhibited

expression of HIF-1 α but not HIF-1 β . Overexpression of HIF-1 α reversed Fara-A-inhibited VEGF transcriptional activation. Our results demonstrated that Fara-A inhibited VEGF transcriptional activation through HIF-1 α expression. Fara-A partly inhibited HIF-1 α mRNA levels. Fara-A blocked the activation of AKT but not of ERK1/2. Overexpression of AKT reversed the Fara-A-inhibited VEGF transcriptional activation, suggesting that Fara-A inhibits VEGF expression via phosphatidylinositol 3-kinase/AKT signaling. These results demonstrate a new function of Fara-A in inhibiting VEGF and HIF-1 α expression and identify a potential molecular mechanism of the regulation.

Ovarian cancer is the leading cause of death from gynecological malignancy and is the most common malignancy affecting women in Western countries: 1 to 2% of all women in these countries develop ovarian cancer at some time during their lives (Bamberger and Perrett, 2002). The disease begins in the peritoneal cavity and is associated with malignant ascites formation. At present, ovarian cancer has the worst prognosis of all gynecological cancers. Cisplatin and its analogs are commonly used for the treatment of ovarian cancer. However, ovarian cancer cells can develop a resistance to cisplatin during treatment, eventually leading to the death of patients from the development of cisplatin-resistant fraction tumors. Therefore, it is important both to identify a new agent with which to treat ovarian cancer and to develop a new therapeutic approach that could be used for the treatment of cisplatin-resistant ovarian cancer.

Angiogenesis (the development of new blood vessels) is

required for tumor growth (Folkman, 2002). Vascular endothelial growth factor (VEGF) is an essential protein for angiogenesis and thus plays an important role in tumor progression (Carmeliet and Jain, 2000; Ferrara, 2002). VEGF mRNA and protein are highly expressed in ovarian tumors and ovarian cancer cell lines (Olson et al., 1994; Abu-Jawdeh et al., 1996). The inhibition of VEGF expression and its receptor function dramatically decreased tumor growth, invasion, and metastasis in animal models. Experimental and clinical data suggests that VEGF is involved in ovarian tumorigenesis and that it plays an important role in tumor-associated ascites formation (Mesiano et al., 1998; Xu et al., 2000). Thus, the possibility of inhibiting angiogenesis by targeting VEGF is an important development in identifying a novel chemotherapeutic approach in treating ovarian cancer.

Most mutations of human cancer are caused by the activation of oncogenes and/or the lost function of tumor suppressor genes, processes that frequently induce angiogenesis and tumor growth. Elevated expressions of VEGF in tumor cells are often induced by environmental factors, such as hypoxia, the activation of oncogenes, or mutations of tumor suppress-

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ABBREVIATIONS: Fara-A, 9- β -D-arabinofuranosyl-2-fluoroadenine; HIF-1, hypoxia-inducible factor 1; PI3K, phosphatidylinositol 3-kinase; VEGF, vascular endothelial growth factor; ERK1/2, extracellular signal-related protein kinase 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRE, hypoxia-responsive element; Luc, Luciferase; β -gal, β -galactosidase; VHL, von Hippel-Lindau; myr-AKT, myristoylated AKT.

sor genes (Mazure et al., 1996; Jiang et al., 1997, 2000; Zundel et al., 2000). Increased VEGF expression can be regulated at the transcriptional level by hypoxia-inducible factor-1 (HIF-1) (Forsythe et al., 1996). HIF-1 is a heterodimeric basic helix-loop-helix transcription factor composed of HIF-1 α and HIF-1 β subunits. HIF-1 binds to a hypoxia-responsive element (HRE) of the VEGF promoter and activates expression of the VEGF gene at the transcriptional level (Forsythe et al., 1996). HIF-1 also activates the transcription of many other genes that are involved in crucial aspects of cancer biology, such as angiogenesis, cell survival, invasion, and glucose metabolism. HIF-1 is over-expressed in many human cancers and the levels of HIF-1 in cells correlate with tumorigenicity (Semenza, 2003). In preclinical studies, the inhibition of HIF-1 activity had marked effects on the reduction of tumor growth.

In most experimental systems, the HIF-1 α protein subunits are constitutively expressed but rapidly degraded by the ubiquitin-proteasome pathway when sufficient oxygen is present (normoxia) (Salceda and Caro, 1997; Kallio et al., 1999). This process is mediated by the specific binding of pVHL, the product of the von Hippel-Lindau (VHL) tumor suppressor gene (Maxwell et al., 1999). The prolyl hydroxylation of HIF-1 α is a critical component in the regulation of HIF-1 α steady-state levels because it controls HIF-1 α -VHL physical interaction (Ivan et al., 2001; Jaakkola et al., 2001). Under hypoxic conditions, the absence of oxygen prevents the hydroxylases from modifying HIF-1 α and thus VHL protein fails to recognize HIF-1 α , thereby allowing HIF-1 α to accumulate (Ivan et al., 2001; Jaakkola et al., 2001).

HIF-1 α expression can be regulated independently of the oxygen environment as well. Growth factors, cytokines, and other signaling molecules stimulate HIF-1 α protein synthesis through activation of the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase pathways (Zhong et al., 2000; Jiang et al., 2001; Fukuda et al., 2002). However, the mechanism of growth factor-induced HIF-1 α expression could be different from that induced by hypoxia. First, hypoxia increases HIF-1 α in all cell types, whereas growth factors stimulate HIF-1 α in a cell-type-specific manner (Semenza, 2003). Second, hypoxia-induced HIF-1 α expression results mainly from a decrease of HIF-1 α degradation, whereas growth factor- and cytokine-induced HIF-1 α expression results from both an increase of protein synthesis and a decrease of degradation by the activation of the PI3K/AKT signaling or mitogen-activated protein kinase pathways. In addition, HIF-1 α expression can be induced by oncogenic mutations, such as VHL lost function, p53 lost function, and PTEN lost function (Maxwell et al., 1999; Ravi et al., 2000; Zundel et al., 2000).

Nucleoside analogs are agents that induce selective DNA damage because they become active upon incorporation into DNA and therefore are specific to S-phase cells (Huang and Plunkett, 1995). 9- β -D-Arabinofuranosyl-2-fluoroadenine (Fara-A), a nucleoside analog, specifically interferes with DNA replication (Huang and Plunkett, 1995). Fara-A has been studied in patients with a variety of lymphoproliferative malignancies and has major anti-tumor effects, especially against chronic lymphoblastic leukemia (Adkins et al., 1997). However, the effectiveness of Fara-A in the treatment of solid tumors is not well known. For the present study, we chose ovarian cancer cell lines

OVCAR-3 and A2780/CP70 as models by which to investigate the effects of Fara-A on ovarian cancer cells. We found that Fara-A significantly inhibited VEGF production through HIF-1 α expression in ovarian cancer cells.

Materials and Methods

Chemicals and Antibodies. Fara-A and other drugs used in this work were from Sigma (St. Louis, MO). Antibodies against HIF-1 α and HIF-1 β were from BD Biosciences (San Diego, CA). The antibodies against phospho-AKT (S473), total AKT, phospho-ERK1/2, and total ERK1/2 were from Cell Signaling (Beverly, MA). The antibodies against p53 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture. The human ovarian cancer cells OVCAR-3 and A2780/CP70 were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 100 units/ml penicillin, and 100 μ g/ml streptomycin in an incubator with 5% CO₂ at 37°C. For hypoxic treatment, the cells were cultured in a chamber containing 1% oxygen, 5% carbon dioxide, and 94% nitrogen and incubated at 37°C.

Plasmid Construction. The VEGF promoter reporter pGL-StuI was constructed by inserting a 2.65-kb fragment of the human VEGF gene promoter into the pGL2 basic luciferase vector as described previously (Forsythe et al., 1996). The VEGF promoter reporter pMAP11wt, containing just 47 bp of VEGF 5'-flanking sequence (from -985 to -939), were cloned by PCR into the pGL2 basic luciferase vector (Forsythe et al., 1996). The mutant reporter pMAP11mut was constructed by introducing a 3-bp substitution at the HIF-1 binding site into pMAP11wt as described previously (Forsythe et al., 1996). Plasmids encoding human HIF-1 α and Myr-AKT were described previously (Ahmed et al., 1997; Jiang et al., 2001).

Immunoblotting Analysis. The ovarian cancer cells were cultured in 60-mm dishes. When the cells reached 80 to 90% confluence, they were treated with Fara-A, then lysed in radioimmunoprecipitation assay buffer (100 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 1 mM leupeptin, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM pepstatin A on ice for 30 min. The supernatant was collected by centrifugation at 12,000g (4°C, 15 min). Protein concentration was determined using protein assay reagent (Bio-Rad, Hercules, CA). Equal amounts of protein were resolved on SDS-PAGE, transferred to a nitrocellulose membrane, and detected by immunoblotting using the specific antibodies. GAPDH was used as a control to indicate the loading and transfer efficiency.

Northern Blotting Analysis. TRIzol reagent (Invitrogen) was used to extract total cellular RNA from cells per the manufacturer's instructions. Cells at 80 to 90% confluence were treated with Fara-A for 6 h. Aliquots of RNA were resolved by electrophoresis in agarose gels (1%), transferred to Hybond-N membranes (Amersham Biosciences), and cross-linked to the membrane by UV radiation. The membrane was probed with ³²P-labeled human VEGF cDNA or human HIF-1 α cDNA. The probes were labeled with [α -³²P]dATP using RadPrime DNA labeling system (Invitrogen) and purified with the ProbeQuant G-50 Micro columns from Amersham Biosciences (Piscataway, NJ). The hybridizations were performed at 42°C in ULTRAhyb hybridization buffer (Ambion, Austin, TX). Messenger RNA of GAPDH was used as an internal control.

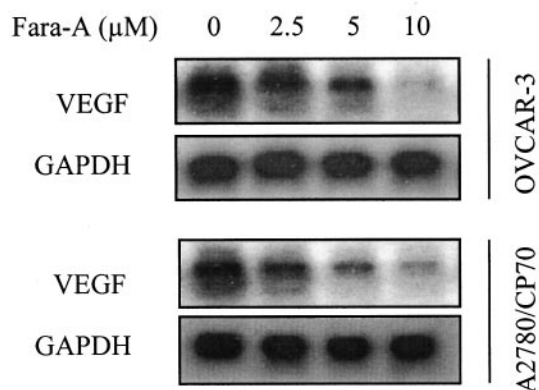
Transient Transfection and Luciferase Activity Assay. OVCAR-3 or A2780/CP70 cells were seeded in six-well plates and cultured to 60 to 80% confluence. The cells were transiently transfected with the VEGF promoter reporter and pCMV- β -galactosidase (β -gal) plasmids using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. At 20 h after transfection, the cells were provided with fresh media with or without Fara-A and incubated for 15 h. Cells were washed once with phosphate-buffered

saline (0.2 g of KH_2PO_4 , 1.16 g of NaH_2PO_4 , 8 g of NaCl , in 1 liter of water, pH 7.0) and lysed with Reporter lysis buffer (Promega, Madison, WI). Luciferase (Luc) activities of the cell extracts were determined using Luciferase assay system (Promega) with a luminometer. The β -gal activity was measured in the assay buffer (50 mM phosphate, pH 7.5, 1 mM MgCl_2 , 50 mM β -mercaptoethanol, and 0.7 mg/ml *o*-nitrophenyl β -D-galactopyranoside). The absorbance at 420

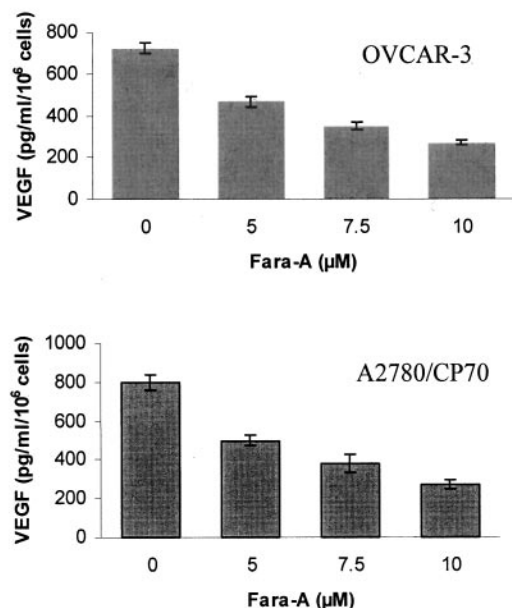
nm was measured with a spectrophotometer. The relative luc activity was calculated as the ratio of luc activity/ β -gal for each sample and normalized to the control in each experiment.

Quantification of VEGF Production. The VEGF production is expressed as VEGF concentration determined in the cell culture supernatants (picograms of VEGF per milliliter per cell number). VEGF in cell culture supernatant was measured using the Quan-

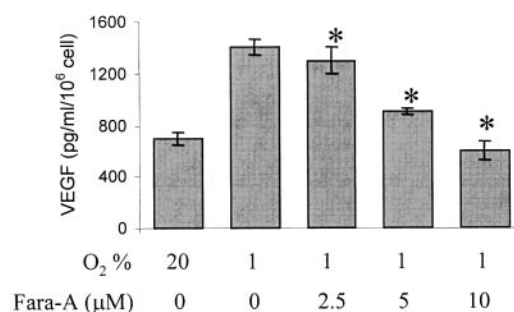
A



B



C



D

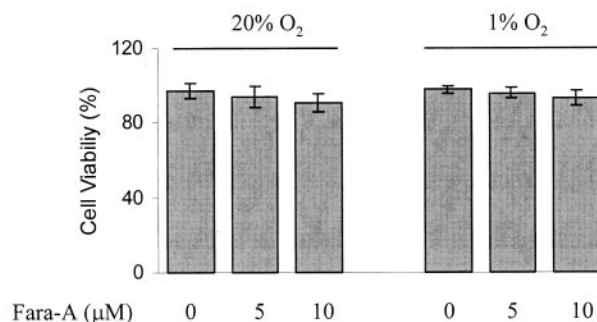


Fig. 1. Fara-A inhibited VEGF expression in OVCAR-3 and A2780/CP70 cells. A, OVCAR-3 and A2780/CP70 cells were seeded in 60-mm plates and cultured to 80 to 90% confluence. The cells were then incubated with Fara-A for 6 h. Total RNA was isolated from the cells, and VEGF mRNA levels were detected by Northern blotting as described under *Materials and Methods*. GAPDH mRNA was used as an internal control. B, OVCAR-3 and A2780/CP70 cells were seeded in 12-well plates. When the cells reached 90 to 100% confluence, the old medium was discarded and fresh medium with or without Fara-A was supplemented. The cells were incubated for 15 h. The medium was collected and subjected to enzyme-linked immunosorbent assay. The data are presented as mean and S.E. from three independent experiments; each experiment was performed with triplicate cultures. C, OVCAR-3 cells at 90 to 100% confluence were provided with fresh medium with or without Fara-A and incubated in the presence of 20% or 1% O_2 (hypoxia) for 15 h. *, $P < 0.05$ versus hypoxia control. The data are presented as mean and S.E. from three independent experiments, and each experiment was performed with triplicate cultures. D, OVCAR-3 cells were seeded in 12-well plates and cultured to 90% confluence, the old medium was discarded and fresh medium with or without Fara-A was supplemented. The cells were cultured in the presence of 20% or 1% O_2 (hypoxia) as described above for 15 h. After incubation under normoxia or hypoxia for 15 h, the numbers of viable cells were determined by Trypan blue exclusion, and the viability of the cells was calculated. The data are presented as mean and S.E. of two experiments, and each experiment was performed with triplicate cultures.

titikine human VEGF enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN), which has been calibrated against a highly purified recombinant human VEGF165. In brief, the cells were seeded in 12-well plates and cultured to 90% confluence. The old medium was discarded and fresh medium was provided with different amounts of Fara-A. In 15 h, the cell culture supernatants were collected and cell numbers of each treatment were counted. VEGF in the supernatant (100 μ l) was determined and normalized to the remaining cell numbers. A serial dilution of human recombinant VEGF was included in each assay to obtain a standard curve.

Trypan Blue Exclusion. Cells were collected and stained with 0.4% of trypan blue for 5 min at room temperature before being examined under the microscope. The number of viable cells was determined by trypan blue exclusion. The dead cells that stained blue were scored positive and counted against the total number of cells to determine the percentage of cell death.

Statistical Analysis. The data represent mean \pm S.E. from three independent experiments except where indicated. Statistical analysis was performed by student *t* test at a significance level of *p* < 0.05.

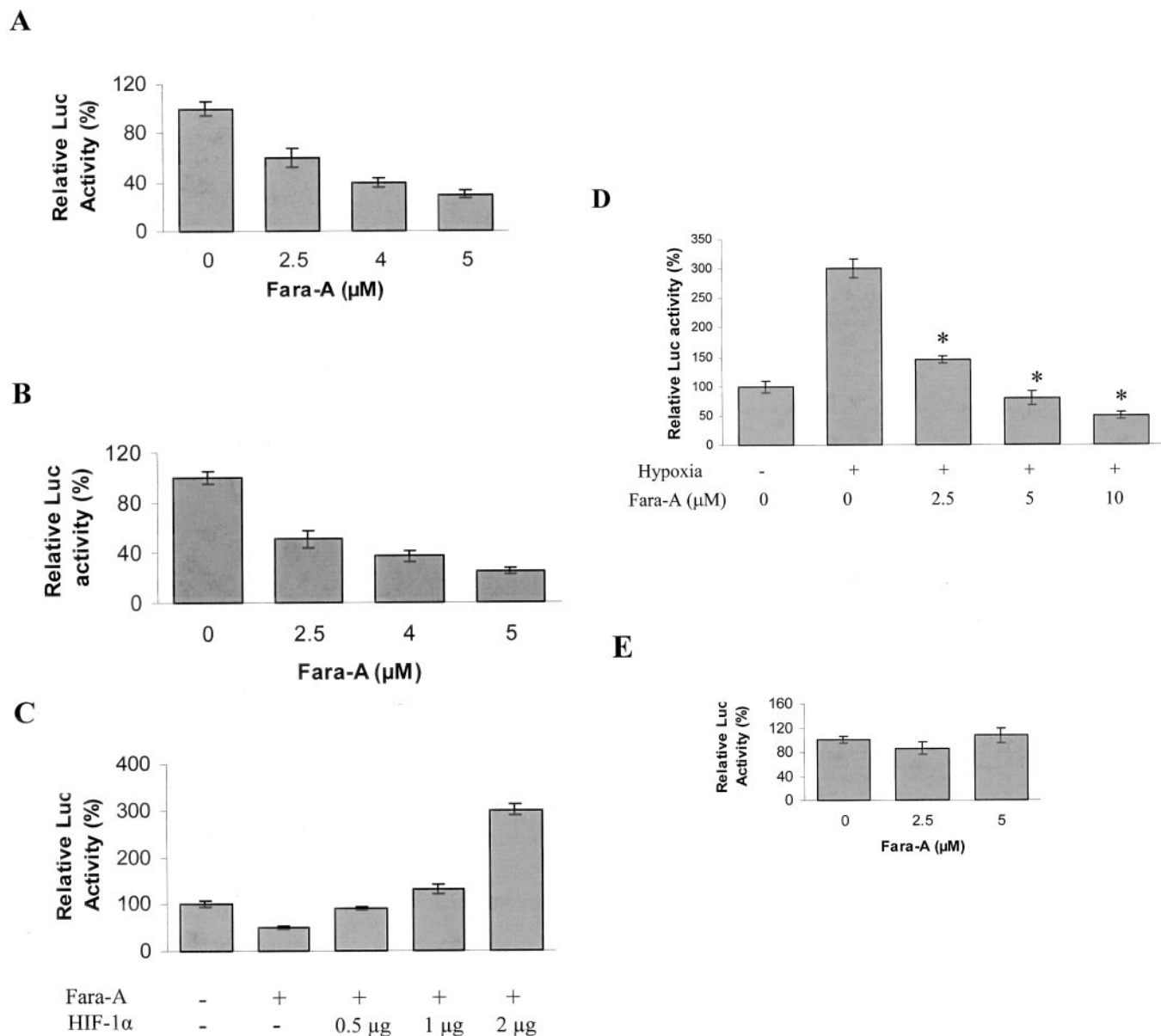


Fig. 2. Fara-A inhibited VEGF transcriptional activation through the HIF-1 DNA binding site. OVCAR-3 cells were seeded in six-well plates and cultured to 60 to 80% confluence for transfection. In these experiments, 1 μ g of each reporter vector and 0.3 μ g of pCMV- β -gal plasmid were used for each well. The empty vector was added to make the equivalent amount of DNA for each treatment. Luc and β -gal activity was determined as described under *Materials and Methods*. The relative Luc activity was defined as the ratio of Luc activity/ β -gal activity and normalized to the control. A, OVCAR-3 cells were transfected with the pGL-StuI VEGF promoter reporter and pCMV- β -gal plasmid. The cells were incubated for 20 h after transfection, followed by a treatment of Fara-A for 15 h as indicated. B, OVCAR-3 cells were transfected with the pMAP11wt VEGF promoter reporter. The cells were incubated for 20 h after transfection and then treated with Fara-A for 15 h. C, OVCAR-3 cells were cotransfected with the pMAP11wt reporter and pCEP4-HIF-1 α plasmid. The cells were incubated for 20 h and then treated with 2.5 μ M of Fara-A for 15 h. D, OVCAR-3 cells were transfected with the p11MAPwt reporter vector and incubated for 20 h. The old medium was discarded and fresh medium was provided with or without Fara-A. The cells were then incubated under hypoxic conditions for 15 h. Reporter activity was determined as described above. *, *P* < 0.05 versus hypoxia control. E, the cells were transfected with a mutant VEGF promoter reporter with a mutation of the HIF-1DNA binding site, pMAP11mut. The data are presented as mean and SE from three independent experiments and each experiment was performed with triplicate cultures.

Results

Fara-A Inhibited VEGF Expression in Ovarian Cancer Cells. To determine the effect of Fara-A on VEGF expression, OVCAR-3 and A2780/CP70 cells were treated with Fara-A, and mRNA levels of VEGF were detected by Northern blotting. As shown in Fig. 1A, the addition of Fara-A decreased VEGF mRNA levels in both OVCAR-3 and A2780/CP70 cells. We next determined the effects of Fara-A on VEGF protein production. OVCAR-3 and A2780/CP70 cells produced a relatively high level of VEGF protein under aerobic conditions (Fig. 1B). The addition of Fara-A decreased VEGF production in both cell lines in a concentration-dependent manner (Fig. 1B). To determine whether Fara-A affected VEGF protein expression under hypoxic conditions, we incubated OVCAR-3 cells with Fara-A in the presence of 20% or 1% O₂. As shown in Fig. 1C, Fara-A abrogated the hypoxia-induced expression of VEGF protein. To test the cell toxicity of Fara-A on the cells, we treated OVCAR-3 cells with 5 and 10 μ M of Fara-A in 20% or 1% O₂ for 15 h, and determined the cell viability by Trypan Blue exclusion method (Fig. 1D). Fara-A treatment did not significantly affect cell viability under the experimental conditions (Fig. 1D), suggesting that the decrease of VEGF protein expression was not caused by the increase of cell death induced by Fara-A.

Fara-A Inhibited VEGF Transcriptional Activation through the HIF-1 DNA Binding Site. To know whether Fara-A inhibits VEGF expression through its transcriptional activation, we determined the effects of Fara-A on the activity of human VEGF promoter reporter pGL2-*StuI* in OVCAR-3 cells. OVCAR-3 cells were transfected with the reporter and β -gal plasmids. Fara-A treatment inhibited the reporter activities in a concentration-dependent manner (Fig. 2A), suggesting that Fara-A inhibits VEGF expression by blocking transcriptional activation of the VEGF gene.

To determine whether Fara-A inhibits VEGF transcriptional activation through the HIF-1 DNA binding site, OVCAR-3 cells were transfected with the pMAP11wt VEGF promoter reporter that contains only the HIF-1 binding site and the adjacent region (Forsythe et al., 1996). As shown in Fig. 2B, the addition of Fara-A significantly inhibited the pMAP11wt reporter activity. In our subsequent experiment, we determined whether forced expression of HIF-1 would restore the Fara-A-inhibited reporter activity. We cotransfected the OVCAR-3 cells with the pMAP11wt reporter and HIF-1 α plasmid. Forced expression of HIF-1 α reversed the Fara-A-inhibited pMAP11wt reporter activity (Fig. 2C). These results suggest that Fara-A inhibits VEGF transcriptional activation through the HIF-1 DNA binding site and

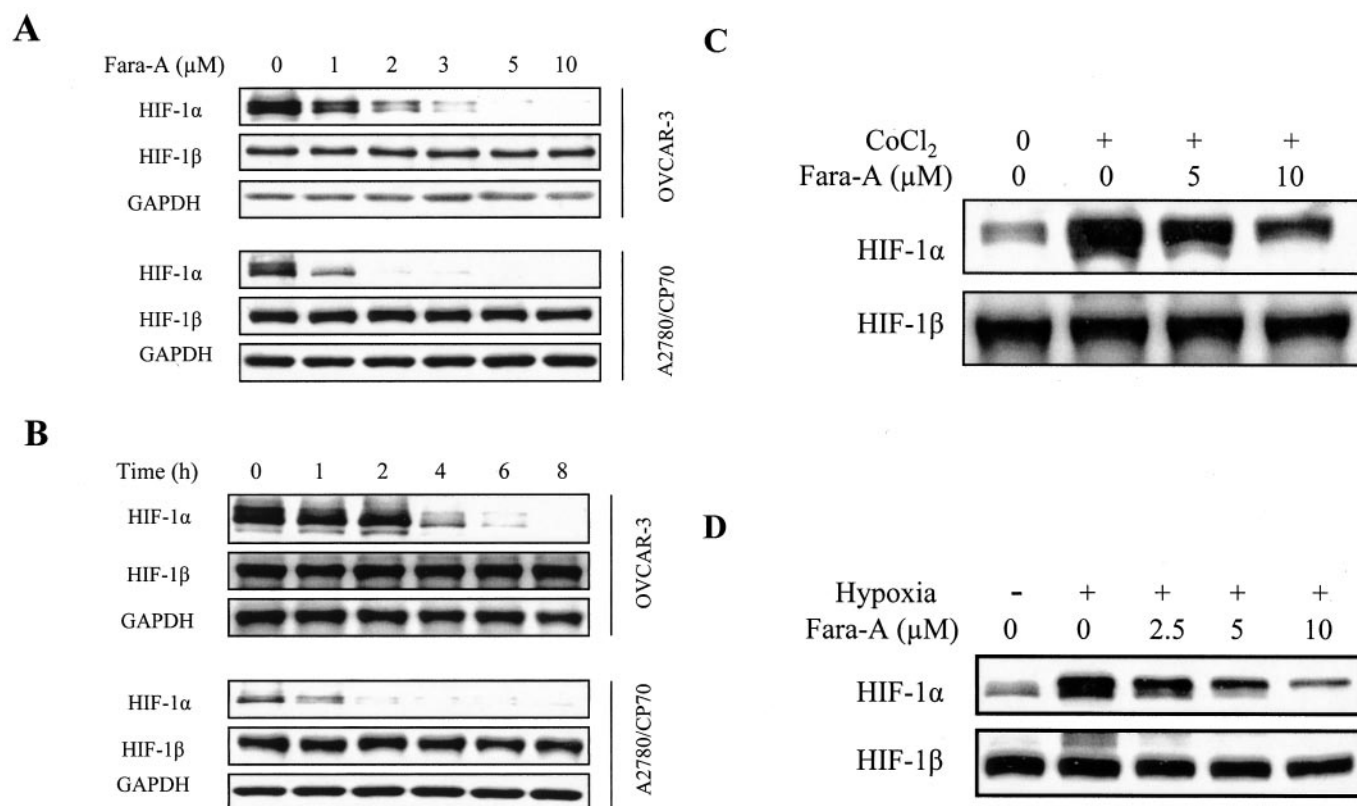


Fig. 3. Fara-A inhibited HIF-1 α expression in ovarian cancer cells. OVCAR-3 and A2780/CP70 cells were seeded in 60 mm plates and cultured to 80 to 90% confluence, followed by treatment with Fara-A as indicated. A, OVCAR-3 cells were treated with various concentrations of Fara-A for 6 h. B, the cells were treated with 4 μ M of Fara-A for different times as indicated. C, the cells were pretreated with Fara-A for 0.5 h followed by treatment with cobalt chloride (75 μ M) for 4 h. D, OVCAR-3 cells were pretreated with Fara-A for 0.5 h and then incubated under hypoxic conditions for 5 h. After treatment, the cells were collected and lysed. Equivalent amounts of protein were resolved on SDS-PAGE and transferred to nitrocellulose membrane. The HIF-1 α and -1 β proteins were detected using specific antibodies as described. GAPDH was used as an internal control to indicate the loading and transfer efficiency. Western blot results of A, B, and D are representative of three different experiments, and the results of C are representative of two different experiments.

HIF-1 α expression. We also determined the effects of Fara-A on VEGF transcriptional activation under hypoxia. As shown in Fig. 2D, hypoxia greatly increased the pMAP11wt reporter activity and Fara-A abrogated the hypoxia-induced VEGF promoter reporter activities. Finally, the mutant VEGF reporter pMAP11mut containing a mutation at the HIF-1 DNA binding site was introduced. The cells were transfected with the pMAP11mut reporter. The luciferase activity was notably less than that of the pMAP11wt reporter, indicating that HIF-1 is a major regulator of VEGF transcriptional activation (data not shown). The addition of Fara-A had little effect on the pMAP11mut reporter activities (Fig. 2E), indicating that Fara-A affects VEGF promoter reporter activity through the HIF-1 DNA binding site. Taken together, these results suggest that Fara-A inhibits VEGF expression at the transcriptional level and through HIF-1 α expression.

Fara-A Inhibited Expression of the HIF-1 α Protein, but Not That of the HIF-1 β Protein. To determine whether Fara-A inhibits VEGF expression through expression of the HIF-1 protein, we determined the effects of Fara-A on HIF-1 α and HIF-1 β protein levels in OVCAR-3 and A2780/CP70 cells. OVCAR-3 and A2780/CP70 cells produced constitutively elevated levels of HIF-1 α protein (Fig. 3, A and B). These results are consistent with recent studies that show ovarian cancer cells such as OVCAR-3 with high basal levels of HIF-1 α under normal culture conditions (Knowles et al., 2003). Fara-A significantly inhibited expression of HIF-1 α protein in both cell lines in a concentration- and time-dependent manner (Fig. 3, A and B). However, Fara-A had little effect on HIF-1 β protein levels in the cells (Fig. 3, A and B). These results suggest that Fara-A inhibits transcriptional activation of VEGF by specifically inhibiting the expression of HIF-1 α but not that of HIF-1 β .

It is well known that hypoxia and cobalt treatment induce HIF-1 α expression. To test whether Fara-A inhibited hypoxia- and cobalt-induced HIF-1 α expression, OVCAR-3 cells were treated with Fara-A in the presence of CoCl₂ or under hypoxic conditions. As shown in Fig. 3C, treatment with Fara-A significantly inhibited the accumulation of HIF-1 α proteins in the presence of cobalt. Likewise, we investigated the effect of Fara-A on HIF-1 α expression under hypoxic conditions. Fara-A treatment inhibited hypoxia-stimulated accumulation of HIF-1 α protein but not expression of HIF-1 β (Fig. 3D). Taken together, these results further demonstrated that Fara-A inhibits VEGF production through HIF-1 α expression induced by hypoxia.

Fara-A Inhibited Expression of HIF-1 α mRNA. Expression of HIF-1 α protein can be regulated through transcriptional and translational mechanisms (Page et al., 2002; Zhang et al., 2003). To identify the possible mechanism through which Fara-A down-regulates expression of the HIF-1 α protein, we determined the effects of Fara-A on HIF-1 α mRNA levels in OVCAR-3 cells. Fara-A decreased the mRNA levels of HIF-1 α to some extent (Fig. 4A). As shown previously in Fig. 3A, our results showed that 5 μ M of Fara-A completely inhibited the expression of HIF-1 α protein in OVCAR-3 cells. However, 5 μ M of Fara-A decreased HIF-1 α mRNA levels by 50% under the same conditions (Fig. 4B), thus indicating that the decrease of HIF-1 α mRNA expression partly accounts for the inhibition of HIF-1 α protein expression. These results suggest that Fara-A inhibits

HIF-1 α expression at both the transcriptional and the translational level.

Fara-A Inhibited AKT Activation but Not ERK1/2 Activation. The PI3K/AKT pathway is involved in the regulation of HIF-1 α expression through protein stability and synthesis. ERK1/2 signaling is also implicated in the regulation of HIF-1 α expression (Richard et al., 1999; Fukuda et al., 2002). To determine whether Fara-A inhibits expression of HIF-1 α through these pathways, we determined the effects of Fara-A on the activation of AKT and ERK1/2 by detecting their phosphorylation levels in the cells. Fara-A inhibited AKT phosphorylation in OVCAR-3 cells (Fig. 5, A and B). However, Fara-A had little effect on the phosphorylation levels of ERK1/2 (Fig. 5, C and D). It was reported recently that p53 could promote the degradation of HIF-1 α protein (Ravi et al., 2000). We observed no effects of Fara-A on the expression of p53 in the ovarian cancer cells under the same experimental conditions, suggesting that Fara-A may not inhibit HIF-1 α expression via p53 expression (data not shown). These results indicate that Fara-A may decrease HIF-1 α expression through the inhibition of AKT activation.

Fara-A Inhibited VEGF Transcriptional Activation through AKT Activity. It has been demonstrated that PI3K/AKT signaling plays an important role in regulating

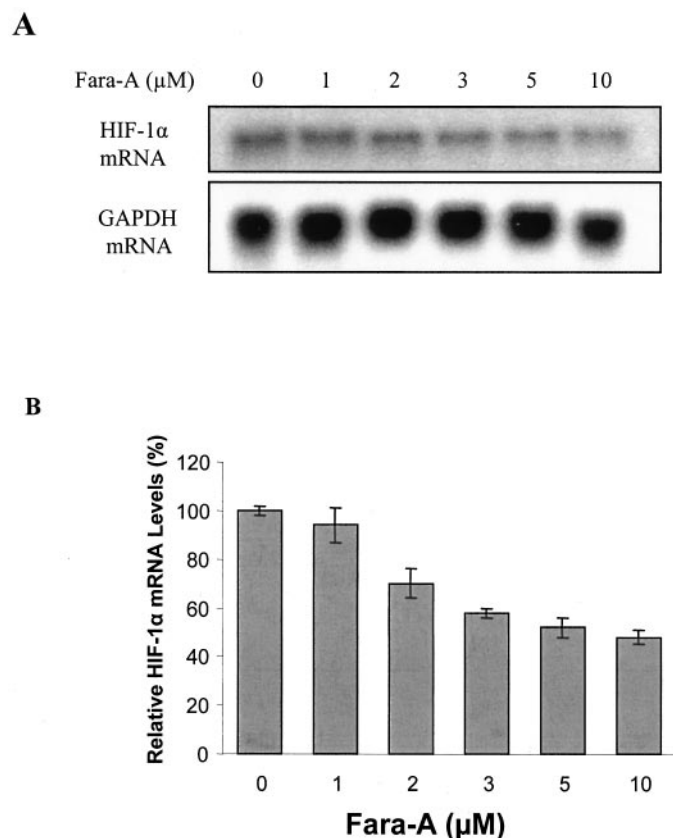


Fig. 4. Effects of Fara-A on HIF-1 α mRNA levels. OVCAR-3 cells were treated with various concentrations of Fara-A (0–10 μ M) for 6 h. Total RNA was isolated from the cells and aliquots of RNA were resolved on 1% agarose gel and transferred to a nylon membrane. A, the membrane was probed with ³²P-labeled human HIF-1 α cDNA. GAPDH mRNA levels were detected and used as loading and transfer efficiency. The immunoblot is representative of three experiments. B, the densities of mRNA bands were measured by densitometry. The relative VEGF mRNA level was calculated as the ratio of VEGF mRNA density/GAPDH mRNA density, and normalized to the control from three replicates.

expression of HIF-1 α and VEGF. In many ovarian cancer cells, the PI3K catalytic subunit was greatly amplified. The PI3K inhibitor LY294002 reduced both the constitutive and inducible expression of HIF-1 α at the mRNA and protein levels and abrogated VEGF expression (Zhang et al., 2003). We also found that LY294002 inhibited activation of AKT and expression of the HIF-1 α protein in both OVCAR-3 and A2780/CP70 cells (data not shown), suggesting that the PI3K/AKT pathway plays a pivotal role in regulating HIF-1 α and VEGF expression. Fara-A significantly inhibited the activation of AKT, indicating that Fara-A may inhibit VEGF expression through AKT activity. To test this possibility, we cotransfected OVCAR-3 cells with the VEGF promoter reporter and Myr-AKT plasmid. As shown in Fig. 5E, transfection of Myr-AKT restored the Fara-A-inhibited VEGF promoter reporter activity. Overexpression of Myr-AKT

increased the levels of phospho-AKT and expression of HIF-1 α protein in OVCAR-3 cells (Fig. 5F). These results suggest that AKT activation is sufficient to reverse Fara-A-inhibited VEGF transcriptional activation and HIF-1 α expression.

Discussion

Several studies have indicated that VEGF regulates angiogenesis, which is an important component of ovarian cancer growth (Mesiano et al., 1998; Xu et al., 2000; Masood et al., 2001). Moreover, VEGF may be an important mediator of ascites formation and tumor metastasis in neoplastic conditions of the ovary (Olson et al., 1994; Mesiano et al., 1998; Xu et al., 2000). It has been shown that VEGF-trap reduced tumor burden and ascites formation related to ovarian cancer (Byrne et al., 2003). Therefore, inhibiting ovarian cancer cell

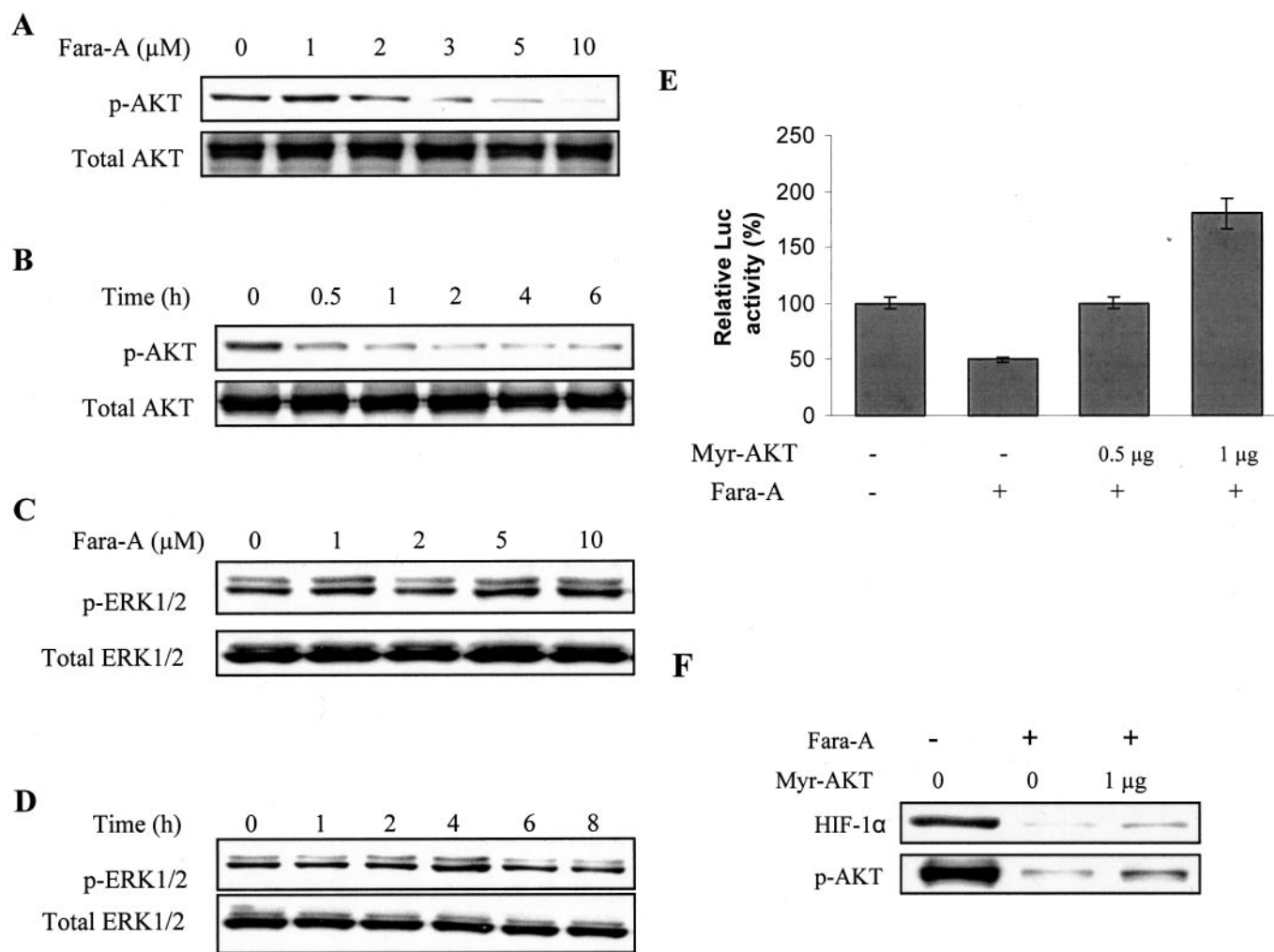


Fig. 5. Fara-A inhibited VEGF expression through inactivation of AKT. **A**, OVCAR-3 cells were seeded in 60-mm dishes and cultured to 80 to 90% confluence. Various concentrations of Fara-A were incubated with the cells for 6 h. Activation of AKT was detected by measuring the phosphorylation of AKT using the specific antibody against phospho-AKT (Ser473). **B**, OVCAR-3 cells were treated with 4 μ M of Fara-A for different times as indicated. **C**, OVCAR-3 cells were incubated with various concentrations of Fara-A for 6 h. The phosphorylation of ERK1/2 was detected by immunoblotting. **D**, OVCAR-3 cells were treated with 4 μ M of Fara-A for different times. ERK1/2 phosphorylation was detected as described above. **E**, OVCAR-3 cells were seeded in six-well plates and cultured to 60 to 80% confluence. Cells were transfected with the pMAP11wt reporter and the Myr-AKT plasmid. The cells were incubated for 20 h, followed by the treatment of 2.5 μ M of Fara-A for 16 h. Relative luc activities were measured as described above. In this experiment, 1 μ g of reporter plasmid and 0.3 μ g of pCMV- β -gal plasmid were used for each well. The empty vector was added to make the equal amount of transfected DNA. The data are presented as mean and S.E. from three different experiments and each experiment was performed with triplicate cultures. **F**, over-expression of AKT restored levels of phospho-AKT and expression of HIF-1 α inhibited by Fara-A in OVCAR-3 cells. The cells were transfected with the empty vector or Myr-AKT plasmid and cultured overnight, then incubated with or without 5 μ M of Fara-A for 6 h. The Western blot results shown here are representative of three experiments.

production of VEGF is an important approach to treating ovarian cancer. We demonstrated in this study that the nucleoside analog Fara-A greatly inhibited VEGF expression in ovarian cancer cells. Fara-A significantly inhibited VEGF expression in ovarian cancer cells under both hypoxic and normoxic conditions (Fig. 1). Fara-A inhibited VEGF expression at a transcriptional level through the HIF-1 DNA binding site (Fig. 2) and through expression of the HIF-1 α protein but not the HIF-1 β protein (Fig. 3). These data suggest that Fara-A inhibits VEGF production through expression of the HIF-1 α protein.

It is known that unless cells have a mutant *VHL* gene, the HIF-1 α protein will be rapidly degraded by the ubiquitin/proteasome pathway under aerobic conditions (Maxwell et al., 1999). Under hypoxic conditions, the ubiquitination of HIF-1 α protein is inhibited and this results in the up-regulation of HIF-1 α protein stability. We found that the human ovarian cancer cells OVCAR-3 and A2780/CP70 expressed relatively high levels of HIF-1 α under aerobic conditions (Fig. 3). Although these ovarian cancer cells do not seem to have a mutant *VHL* gene (Foster et al., 1995), the cells do have high levels of PI3K and AKT activity, which may be involved in maintaining the constitutively elevated levels of HIF-1 α . Studies addressing the role of PI3K signaling in hypoxia-induced HIF-1 α expression were contradictory based on the cell lines used. In a study of prostate cancer cells, it was observed that PI3K and AKT activity was required for HIF-1 α expression (Zhong et al., 2000; Hudson et al., 2002). However, in 1c1c7 mouse hepatocyte cells, the inhibition of PI3K activity did not affect hypoxia-induced HIF-1 α expression (Arsham et al., 2002). These different results could be caused by cell type-specific effects. Several recent studies using different cell systems have demonstrated that the levels of HIF-1 α expression were induced by growth factors and oncogenes through the activation of PI3K/AKT signaling (Zhong et al., 2000; Zundel et al., 2000; Jiang et al., 2001; Fukuda et al., 2002; Gao et al., 2002). p110 α , the catalytic subunit of PI3K, is increased in copy numbers in approximately 40% of ovarian cancer cells (Shayesteh et al., 1999), and the PI3K catalytic subunit expression positively correlated with the expression of VEGF in ovarian cancer (Zhang et al., 2003). These results suggest that PI3K/AKT signaling may play an important role in regulating HIF-1 α expression in ovarian cancer cells. The activation of PI3K/AKT signaling may account for the increase of HIF-1 α expression. The mutation of other tumor suppressor genes, such as p53, may also induce HIF-1 α protein expression (Ravi et al., 2000). It was reported that the OVCAR-3 cells expressed a mutant p53 protein (Yaginuma and Westphal, 1992) and that the A2780/CP70 cells showed a loss of p53 function (Brown et al., 1993), which may also account for the high basal levels of HIF-1 α protein in these cells. Fara-A significantly inhibited activation of AKT in ovarian cancer cells (Fig. 5, A and B), which accounts for the down-regulation of HIF-1 α . We found that Fara-A inhibited expression of HIF-1 α protein under both normoxia and hypoxia conditions (Fig. 3, A, B, and D). Fara-A treatment also inhibited the cobalt chloride-induced expression of HIF-1 α protein in the cells (Fig. 3C).

Ovarian cancer is the leading cause of death from gynecological malignancy and the fourth most common cause of cancer death among American women. Cisplatin and its an-

alogs are commonly used for the treatment of ovarian cancer; however, during treatment, ovarian cancer tumors can become resistant to cisplatin, causing many patients to die as a result of drug-resistant fraction tumors. Therefore, it is crucial to identify a new agent that will treat ovarian cancer and to develop a new therapeutic approach that could be used for the treatment of cisplatin-resistant ovarian cancer. An anti-angiogenesis approach is one promising method for inhibiting tumor growth. One way to inhibit tumor angiogenesis is to inhibit the production of VEGF in cancer cells. HIF-1 activates the transcription of the VEGF gene and many other genes that are involved in tumor growth and angiogenesis (Semenza, 2003) and is an emerging target for cancer therapy. Fara-A has been studied in patients with a variety of lymphoproliferative malignancies and has major antitumor effects, especially against chronic lymphoblastic leukemia (Adkins et al., 1997). Clinical experience indicates that Fara-A is an effective and generally well tolerated antineoplastic agent (Adkins et al., 1997). However, the effects of Fara-A on solid tumors are poorly understood. We demonstrated in this study for the first time that the nucleoside analog Fara-A inhibited expression of HIF-1 α and VEGF in cisplatin-resistant OVCAR-3 and A2780/CP70 cancer cells. Our results suggest a potential use of this drug in treating ovarian cancer in the future.

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