**Aminoflavone, a Ligand of the Aryl Hydrocarbon Receptor, Inhibits HIF-1α Expression in an AhR-Independent Fashion**

Erika Terzuoli1,2, Maura Puppo1,3, Annamaria Rapisarda1, Badarch Uranchimeg1, Liang Cao4, Angelika M. Burger5, Marina Ziche2, and Giovanni Melillo1

**Abstract**

Aminoflavone (AF), the active component of a novel anticancer agent (AFP464) in phase I clinical trials, is a ligand of the aryl hydrocarbon receptor (AhR). AhR dimerizes with HIF-1β/AhR, which is shared with HIF-1α, a transcription factor critical for the response of cells to oxygen deprivation. To address whether pharmacologic activation of the AhR pathway might be a potential mechanism for inhibition of HIF-1, we tested the effects of AF on HIF-1 expression. AF inhibited HIF-1α transcriptional activity and protein accumulation in MCF-7 cells. However, inhibition of HIF-1α by AF was independent from a functional AhR pathway. Indeed, AF inhibited HIF-1α expression in AhRKO cells, in which the AhR pathway is functionally impaired, yet did not induce cytotoxicity, providing evidence that these effects are mediated by distinct signaling pathways. Moreover, AF was inactive in MDA-MB-231 cells, yet inhibited HIF-1α in MDA-MB-231 cells transfected with the SULT1A1 gene. AF inhibited HIF-1α mRNA expression by ~50%. Notably, actinomycin-D completely abrogated the ability of AF to downregulate HIF-1α mRNA, indicating that active transcription was required for the inhibition of HIF-1α expression. Finally, AF inhibited HIF-1α protein accumulation and the expression of HIF-1 target genes in MCF-7 xenografts. These results show that AF inhibits HIF-1α in an AhR-independent fashion, and they unveil additional activities of AF that may be relevant for its further clinical development.

**Introduction**

Hypoxia, a decrease in oxygen levels, is a hallmark of solid tumors. The response of mammalian cells to hypoxia is mediated, at least in part, by a family of transcription factors known as hypoxia inducible factors (HIF; ref. 1). HIF-1α is a heterodimer consisting of a constitutively expressed β subunit and an oxygen regulated α subunit (2), which is ubiquitinated and degraded under normoxic conditions (3). In contrast, under hypoxic conditions, the HIF-α subunit is stabilized and translocates to the nucleus, in which it dimerizes with HIF-1β (also known as AhR nuclear translocator) and activates the transcription of genes involved in key steps of tumorigenesis, including angiogenesis, metabolism, proliferation, metastasis, and differentiation (4). Overexpression of HIF-α has been reported in >70% of human cancers (5–11) and is associated with poor patient prognosis, making HIF-1 an attractive target for the development of novel cancer therapeutics (12). Aminoflavone (AF; NSC 686288) is the active component of a produg (AFP464) in phase I clinical trials. AFP464 is rapidly converted to AF, in plasma or tissue culture, by nonspecific plasma esterases. AF has a unique COMPARE pattern of cytotoxicity in the NCI60 (13, 14), with activity only in a subset of cell lines, including MCF-7 breast cancer cells (15–18). The sensitivity of cancer cell lines to AF has been associated with its ability to act as a ligand of the aryl hydrocarbon receptor (AhR), which upon dimerization with HIF-1β/AhR nuclear translocator activates transcription by binding to the xenobiotic response element (XRE) in the promoters of target genes, including but not limited to cytochrome P450 1A1 (CYP1A1). Indeed, the presence of a functional AhR pathway and the induction of CYP1A1 expression by AF seem to be essential for its antiproliferative activity in MCF-7 cells (18–20).

Several studies have suggested the existence of a cross-talk between the AhR and HIF-1 pathways (21–26). However, whether pharmacologic activation of the AhR pathway may be a viable approach to inhibit HIF-1 remains poorly understood. We show that AF inhibits HIF-1α expression, both in vitro and in MCF-7 xenografts, in an AhR-independent manner.
fashion. Notably, AF partially inhibited HIF-1α mRNA expression, yet almost completely blocked HIF-1α protein accumulation. The ability of actinomycin-D to completely revert inhibition of HIF-1α mRNA expression by AF is consistent with the existence of a transcription-dependent pathway that may regulate HIF-1α mRNA expression and its translation.

**Materials and Methods**

**Cell lines and reagents**

Human cell lines were maintained in RPMI 1640 supplemented with L-glutamine and 5% heat-inactivated fetal bovine serum (Hyclone), and grown at 37°C in 5% CO2 and ambient oxygen (normoxia). Hypoxia was achieved in an InVivo2 400 hypoxic workstation (Ruskinn Technology) delivering 1% oxygen in 5% CO2 at 37°C. AhR-deficient MCF-7 cells (AhR<sup>R100</sup>; ref. 27) were kindly provided by Dr. David Vistica [STB, National Cancer Institute (NCI), Frederick, MD]. MDA-MB-231 cells stably transfected with a SULT1A1 cDNA, MDA/SULT1A1 (28), were kindly provided by Dr. David C. Spink, (Wadsworth Center, Albany, NY). All cell lines were obtained from the Developmental Therapeutics Program and were validated according to information provided on the Developmental Therapeutics Program Web site (http://dtp.nci.nih.gov/branches/btb/characterizationNCI60.html). AF (NSC 686288) was from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI.

**Sulforhodamine B assay**

Cells, seeded into 96-well plates, were treated with AF for an additional 72 hours, and cell viability was assessed as previously described (29).

**Immunoblot analysis**

Total protein lysates were obtained as previously described (30). Antibodies used are as follows: HIF-1α and p21 (BD- Transduction Laboratories); HIF-1β, SULT1A1, HIF-2α, and AhR (Novus Biologicals); actin (Chemicon International); and γH2AX (Cell Signaling, Inc.).

**Real-time PCR**

Vascular endothelial growth factor (VEGF), HIF-1α, p21, CA9, LOX, actin, and CYP1A1 expression was measured by real-time PCR as previously described (30). Primers and probes used are listed in Supplementary Table S1 and Table 1. VEGF primers and probe were previously described (30). 18S rRNA was used as an internal control.

**HIF-1α protein translational assay**

MCF-7 cells, treated for 12 hours with AF (0.25 μmol/L), were labeled with 35S-methionine/cysteine (MP Biomedicals) as previously described (31). Total 35S incorporation was monitored by both size fractionation of total lysates and TCA precipitation.

**Luciferase activity**

MCF-7 cells were transfected using Effectene (Qiagen) with pGL2-TK-HRE, pGL3-control (30), NF-κB-Luc, activator protein (AP-1)-luc (supplied by Dr. Nancy Colburn, NCI, Frederick, MD), and XRE-Luc (supplied by Dr. F. J. Gonzalez, NCI, NIH, Bethesda, MD). Efficiency of transfection was assessed by cotransfection with pQTK-RENilla (Promega). Results are expressed as fold increase of luciferase levels relative to normoxic nontreated controls.

**Transfection with small interfering RNA for HIF-1α, SULT1A1, and AhR**

Small interfering RNA (siRNA) targeting SULT1A1 (s5′-CA-GAGGGAGTGTGCGAATCAA), AhR (s5′-TTGAGTATATAGTTTGTGA), HIF-1α (s5′-AGGACAAGTCACCACAGGA), and negative control were purchased from Qiagen.

**Animal studies**

Studies were conducted in a facility that is accredited by the Association of Assessment and Accreditation of Laboratory Animal Care with an approved animal protocol. MCF-7 cells (1 × 10<sup>7</sup>) were injected s.c. into the flank of female athymic nude (NCr/nu) mice (Animal Production Area, NCI, Frederick, MD). β-Estradiol cypionate (3 mg/kg) was administered to mice on estrus, and tumors were allowed to grow for 3 weeks before initiating treatment.

**Table 1. Primers used for real-time PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CYP1A1</td>
<td>5′-ATTGGGGCATCATGCTGACG-3′</td>
<td>5′-TGCTTGCTCATCCTTGACAG-3′</td>
<td>5′-TTGGATTAAAT-GAGGCCTGGCCGTGTTG-3′</td>
</tr>
<tr>
<td>Human p21</td>
<td>5′-ACGCGACTGTGATGGCGC-3′</td>
<td>5′-AAAGTTCATCAGCTCAAGGGG-3′</td>
<td>5′-AAGGACAAGTCACCACAGGA-3′</td>
</tr>
<tr>
<td>Human CA 9</td>
<td>5′-GAGGCCCTGGCCGTGTG-3′</td>
<td>5′-AATCACGTGAGGGAAGGAAA-3′</td>
<td>5′-TTGCACTGCACAGGCCACATTCAC-3′</td>
</tr>
<tr>
<td>Human LOX</td>
<td>5′-TGCTTGTTGGAGACTGAGATACC-3′</td>
<td>5′-AATCACTGTGAGGGGAAGGAGAAA-3′</td>
<td>5′-TTGGATTAAAT-GAGGCCTGGCCGTGTTG-3′</td>
</tr>
<tr>
<td>Human HIF-1α (intron 5-exon 6)</td>
<td>5′-TGCTTTTTTTTTTCCCTGACATTG-3′</td>
<td>5′-TTGTTTATCCTGGTGATGCATACCATACGGA-3′</td>
<td>5′-TAGCCGAGGAGAAGAATGACATGAA-3′</td>
</tr>
<tr>
<td>Human HIF-1α (exon 5-exon 6)</td>
<td>5′-TTGAGGTGTTACCTGGTGATGCATACCATACGGA-3′</td>
<td>5′-TTGAGGTGTTACCTGGTGATGCATACCATACGGA-3′</td>
<td>5′-TTGAGGTGTTACCTGGTGATGCATACCATACGGA-3′</td>
</tr>
</tbody>
</table>
administered i.m. every 7 days. Tumor size was determined by collecting length and width measurements, and calculating the tumor weight (mg) as [tumor length × (tumor width)^2]/2, in which the tumor length is the longest dimension (mm) and the tumor width is the narrowest dimension (mm). AF (saline/0.05% Tween 80) was dosed i.p. Five mice per group were treated daily for 4 days with AF (60 mg/kg) or vehicle control. When mice were sacrificed (day 4), tumors from each animal were harvested and used to analyze mRNA and protein expression, as previously described (32).

Tissue HIF-1α assay
Tissue lysates were prepared as previously described (32) and used for the quantitative determination of HIF-1α using electrochemiluminescence assay (Meso-Scale). HIF-1α concentration (pg/mL) was determined using a recombinant protein (R&D Systems) as standard.

Statistical analysis
Results are either representative or average of at least three independent experiments done. Statistical analysis was performed using ANOVA test and t test (Prism, GraphPad).

Results
AF inhibits HIF-1α transcriptional activity in MCF-7 breast cancer cells
To test whether AF inhibited HIF-1 activity, MCF-7 cells were transiently transfected either with pGL2-TK-HRE, containing the luciferase reporter gene under control of three copies of a hypoxia response element (HRE) or with a control vector (pGL3 control). Hypoxia increased HRE-dependent luciferase expression by 49-fold, relative to cells cultured under normoxic conditions (Fig. 1A, left). AF inhibited luciferase expression in a dose-dependent manner, but did not affect constitutive luciferase expression (Fig. 1A, right), suggesting that inhibition of luciferase by AF was HIF-1-dependent. AF also inhibited endogenous HIF-1 transcriptional activity, as indicated by inhibition of hypoxic induction of VEGF (Fig. 1B, left), CA9, and LOX mRNA expression (Fig. 1B, right), similar to the effects of siRNA targeting HIF-1α. In contrast, AF caused up to 40-fold increase of p21 mRNA expression, up to 200-fold higher levels of CYP1A1 mRNA expression in MCF-7 cells cultured under either normoxia (data not shown) or hypoxia (Fig. 1C, right), consistent with the induction of p21 mRNA expression and showing a differential effect on distinct target proteins.

AF inhibits HIF-1α and HIF-2α protein accumulation in a cell type–dependent fashion
To address whether AF inhibited HIF-1α and HIF-2α protein accumulation, we tested six cell lines from the NCI60 panel that were reported to be sensitive to AF. Hypoxia increased HIF-1α protein expression in all the cell lines examined, whereas HIF-2α was induced to detectable levels in three cell lines (T47D, CAKI, and UACC257). AF (0.5 μmol/L) inhibited HIF-1α protein accumulation, albeit to a different extent, in MCF-7, T47D, CAKI, and OVCAR3. In contrast, HIF-2α was slightly decreased by AF in T47D and CAKI cells but not in UACC257 (Fig. 2A), suggesting that AF inhibits HIF-1α and HIF-2α protein accumulation in a cell type–specific fashion, although HIF-2α seems to be slightly less susceptible to AF inhibition, relative to HIF-1α.

Further experiments showed that AF inhibited HIF-1α protein accumulation in MCF-7 cells in a dose-dependent fashion, with ~80% decrease at 0.25 μmol/L (Fig. 2B). By contrast, HIF-1β was only inhibited by 15%, and actin levels were not changed in the presence of up to 1 μmol/L of AF. Kinetic experiments showed that AF (0.25 μmol/L) caused little or no inhibition of HIF-1α protein at 8 hours, but completely abrogated its accumulation after 12 to 24 hours of treatment (Fig. 2C). AF also inhibited HIF-1α protein accumulation induced by the iron chelator desferrioxamine (100 μmol/L; Fig. 2D), suggesting that its effects are not restricted to hypoxic signaling. By contrast, AF induced p21 protein accumulation in MCF-7 cells cultured under either normoxia (data not shown) or hypoxia (Fig. 1C, right), consistent with the induction of p21 mRNA expression and showing a differential effect on distinct target proteins.

A functional AhR pathway is not required for the inhibition of HIF-1α expression by AF
Previous studies have indicated that AF is a ligand of AhR (18). Indeed, AF caused a 7- to 8-fold increase in XRE-dependent luciferase expression (Fig. 3A) and induced up to 200-fold higher levels of CYP1A1 mRNA expression in MCF-7 cells (Fig. 3B), showing that AF was able to induce AhR-dependent transcriptional activity. TCDD, used as positive control, induced a 15-fold increase in XRE-dependent luciferase expression (Fig. 3A) and up to 1,160-fold higher levels of CYP1A1 mRNA expression (Fig. 3B), relative to nontreated cells.

To address whether inhibition of HIF-1α by AF required a functional AhR pathway, we took advantage of AhR100 cells, MCF-7–derived cells that express low levels of AhR and are resistant to the cytotoxic effects of AF (18). Indeed, AF caused cytotoxicity in parental MCF-7 cells but not in AhR100 cells, even at concentrations as high as 2 μmol/L (Fig. 3C). Consistent with a functional impairment of the AhR pathway, TCDD induction of XRE-dependent luciferase expression was decreased by 75% in AhR100 cells, relative to parental MCF-7 cells, and induction of CYP1A1 mRNA expression was decreased by 95% (Fig. 3A and B). More importantly, AF failed to induce XRE-dependent luciferase expression in AhR100 cells and only modestly induced CYP1A1 mRNA expression (35% of the levels induced in wild-type MCF-7 cells; Fig. 3A and B). However, AF inhibited hypoxic induction of HIF-1α transcriptional activity irrespective of a functional AhR, as shown by the inhibition of HRE-dependent luciferase expression in MCF-7 and AhR100 cells (Fig. 3D). Accordingly, AF also completely inhibited hypoxic induction of HIF-1α protein accumulation in AhR100 cells (Fig. 3E), showing that inhibition of HIF-1α expression by AF is independent from a functional AhR pathway.
AF inhibits HIF-1α transcriptional activity. A, MCF-7 cells were transfected with pGL2-TK-HRE (left) or pGL3-Control (right), and cultured under normoxia or hypoxia in the absence or presence of increasing concentrations of AF. B, MCF-7 cells were cultured under normoxia or hypoxia for 16 h in the absence or presence of increasing concentrations of AF, and VEGF mRNA levels were measured (left). Right, MCF-7 cells were cultured under normoxic or hypoxic conditions for 16 h either in the absence or presence of AF (0.25 μmol/L) or after transfection with a siRNA targeting HIF-1α (inset, HIF-1α downregulation following siRNA transfection). Levels of LOX and CA9 mRNA are expressed as fold increase relative to nontreated normoxic controls. C, MCF-7 cells were cultured under normoxic or hypoxic conditions for 16 h in the absence or presence of AF (0.25 μmol/L). Levels of p21 mRNA and protein were assessed. D, MCF-7 cells, transfected with NF-κB-luc (left) or AP-1-luc (right), were treated for 16 h under normoxic or hypoxic conditions in the absence or presence of AF (0.25 μmol/L), tumor necrosis factor α (30 ng/mL), or 12-O-tetradecanoylphorbol-13-acetate (10 ng/mL), as indicated.
AF inhibits HIF-1α in MDA/SULT1A1, but not in MDA-MB-231 parental breast cancer cells

The restricted spectrum of AF activity in the NCI60 cells has been attributed to a requirement for its intracellular activation (18) by pathway(s) yet to be completely elucidated. A potential correlation between sensitivity to AF and expression of SULT1A1 has also been suggested, consistent with the ability of SULT1A1 to induce the formation of AF metabolites that mediate DNA damage (33). Indeed, AF exerted antiproliferative effects in MDA/SULT1A1, but not in MDA-MB-231 parental cells (Fig. 3C). However, both MDA-MB-231 and MDA/SULT1A1 cells express little or no AhR transcriptional activity, as indicated by minimal if any induction of XRE-dependent luciferase (Fig. 3A) or CYP1A1 mRNA expression by either AF or TCDD (Fig. 3B).

We then tested whether AF inhibited HIF-1 transcriptional activity in MDA-MB-231 and MDA-SULT1A1 cells. AF completely inhibited HIF-1–dependent luciferase expression in MDA/SULT1A1, but did not affect its expression in MDA-MB-231 parental cells, showing that exogenous expression of SULT1A1 was sufficient to mediate AF-dependent inhibition of HIF-1 activity (Fig. 3D). Accordingly, AF almost completely inhibited hypoxic induction of HIF-1α protein in MDA/SULT1A1 cells, but not in MDA-MB-231 (Supplementary Fig. S1; Fig. 3E).

Next, we evaluated expression of HIF-1α in MCF-7 cells transfected with either negative control siRNA or siRNA targeting SULT1A1 or AhR (Supplementary Fig. S2A-B). AF inhibited hypoxic induction of HIF-1α protein by 75% in cells transfected with negative control siRNA and by 50% in cells transfected with AhR siRNA, relative to hypoxia-treated cells (Supplementary Fig. S2B). In contrast, downregulation of SULT1A1 almost completely prevented the inhibition of HIF-1α by AF (15% inhibition, compared with hypoxia; Supplementary Fig. S2B), showing that SULT1A1 expression was implicated in HIF-1α inhibition by AF and further supporting the conclusion that inhibition of HIF-1α by AF is independent from a functional AhR pathway.

**Inhibition of HIF-1α by AF is independent from DNA damage**

Induction of DNA damage by AF, measured by phosphorylation of H2AX, paralleled results obtained in cytotoxicity assay.
Indeed, AF induced significantly higher levels of γH2AX in sensitive MCF-7 and MDA/SULT1A1 cells than in resistant AhR100 and MDA-MB-231 cells (Fig. 3E). However, AF was equally able to inhibit hypoxic induction of HIF-1α protein in AhR100 and MDA/SULT1A1 cells, showing a complete dissociation between induction of DNA damage and HIF-1α inhibition (Fig. 3E).

We have previously shown that agents that inhibit topoisomerase I or II may affect HIF-1α protein translation (31, 34). Consistent with the finding that induction of DNA damage by AF does not seem to involve topoisomerases (35), AF was able to inhibit HIF-1α protein expression in cells transfected with siRNA targeting topo I or topo IIα.

Figure 3. AF inhibits HIF-1α in an AhR-independent fashion. A, MCF-7, AhR100, MDA-MB-231, and MDA/SULT1A1 were transfected with XRE-luc and then treated with TCDD (1 μmol/L) or AF (0.25 μmol/L) for 16 h. ***, P < 0.001, relative to control. B, MCF-7, AhR100, MDA-MB-231, and MDA/SULT1A1 were cultured under normoxia for 16 h in the absence or presence of AF (0.25 μmol/L) or TCDD (1 μmol/L). CYP1A1 mRNA expression was analyzed. ***, P < 0.001, relative to control. C, MCF-7, AhR100, MDA-MB-231, and MDA/SULT1A1 were treated with increasing concentrations of AF as indicated for 72 h. Cell viability was assessed. D, MCF-7, AhR100, MDA-MB-231, and MDA/SULT1A1 were transfected with pGL2-TK-HRE and then treated for 16 h under normoxic or hypoxic conditions in the absence or presence of AF (0.25 μmol/L). ***, P < 0.001; **, P < 0.005, relative to hypoxia. E, MCF-7, AhR100, MDA-MB-231, and MDA/SULT1A1 were cultured under normoxia or under hypoxic conditions in the absence or presence of AF (0.25 μmol/L) for 16 h. Levels of HIF-1α, actin, and γH2AX were measured.
Furthermore, AF inhibited the hypoxic induction of HIF-1α protein accumulation in the presence of aphidicolin, which blocks DNA polymerase and prevents DNA damage (Supplementary Fig. S3), further showing that AF inhibited HIF-1α by a DNA damage–independent pathway.

**AF does not affect HIF-1α degradation, but decreases the rate of HIF-1α translation**

HIF-1α steady-state is the result of a balance between protein translation and its degradation. To investigate whether AF affected HIF-1α degradation, we performed experiments in the presence of inhibitors of either proteasome activity (MG-132) or prolyl hydroxylase enzymes (DMOG, a global inhibitor of 2-oxoglutarate–dependent dioxygenase enzymes), which cause an accumulation of HIF-1α under normoxic conditions. As shown in Fig. 4A, AF inhibited HIF-1α protein accumulation regardless of inhibition of the proteasome or prolyl hydroxylases, suggesting that AF did not affect degradation of HIF-1α protein. Consistent with these results, AF also inhibited normoxic accumulation of HIF-2α in a sensitive Von Hippel-Lindau (VHL)–deficient renal cancer cell line (A498, which does not express HIF-1α), indicating that a functional VHL pathway, essential for normoxic degradation of the HIF-α subunit, is not required for the inhibition of HIF-α by AF (Fig. 4B). Finally, experiments conducted in the presence of cycloheximide failed to show significant differences in HIF-1α protein half-life between cells cultured in the absence or presence of AF (half-life ~60 min; Fig. 4C). Taken together, these results show that AF does not affect HIF-1α protein degradation.

We then assessed whether AF inhibited HIF-1α translation by evaluating the rate of HIF-1α protein synthesis in normoxic MCF-7 cells in the absence or presence of AF. Cells were pulse labeled with [35S] methionine for 60 minutes at which point HIF-1α was immunoprecipitated and analyzed by PAGE and autoradiography. As shown in Fig. 4D (left), AF significantly decreased 35S-labeled HIF-1α accumulation relative to nontreated cells, suggesting that it may affect its rate of translation. Importantly, under the same experimental conditions, AF did not affect degradation of HIF-1α protein.
conditions AF did not significantly affect the synthesis of HIF-1β or that of total proteins (Fig. 4D, right).

Active transcription is required for the inhibition of HIF-1α mRNA expression by AF

Regulation of HIF-1α translation under hypoxic conditions is still poorly understood, and recent evidence suggests that levels of HIF-1α mRNA may be a crucial factor affecting the rate of HIF-1α translation (36). AF, under hypoxic but not normoxic conditions, caused a 50% decrease of HIF-1α mRNA expression, relative to normoxic cells (P = 0.005; Fig. 5A). Next, to address whether AF inhibited HIF-1α mRNA transcription, we measured the levels of HIF-1α pre-mRNA. Unexpectedly, we found that hypoxia induced up to 5.8-fold higher levels of
HIF-1α pre-mRNA, relative to normoxic cells (Fig. 5B). Notably, despite the increase of HIF-1α pre-mRNA under hypoxic conditions, which was confirmed using a different set of primers (data not shown), hypoxia only marginally affected the levels of HIF-1α mRNA, suggesting possible abnormalities in HIF-1α mRNA maturation and/or processing. AF did not affect HIF-1α pre-mRNA expression under normoxic conditions, yet reduced the hypoxic induction to levels 2-3-fold higher than those present in normoxic cells.

To further address the mechanism by which AF affected HIF-1α mRNA levels, we performed experiments in the presence of actinomycin-D, an inhibitor of transcription. We found that hypoxic induction of HIF-1α pre-mRNA, both in the absence and presence of AF, was completely abrogated by addition of actinomycin-D (Fig. 5D), showing that it was dependent on active transcription. Surprisingly, we also found that inhibition of mature HIF-1α mRNA expression by AF, observed in hypoxic cells, was completely reversed by addition of actinomycin-D, showing that induction rather than inhibition of transcription is required for HIF-1α mRNA downregulation in the presence of AF (Fig. 5C). Consistent with these results, addition of actinomycin-D also partially reversed the inhibitory effect of AF on HIF-1α protein accumulation (Supplementary Fig. S3), suggesting that the effects on HIF-1α mRNA expression were causally related to the inhibition of HIF-1α protein.

Taken together, these results show that active transcription, in hypoxic cells treated with AF, is required for the inhibition of HIF-1α mRNA expression.

**AF inhibits HIF-1α expression in MCF-7 xenografts**

To test whether AF was able to inhibit HIF-1α expression in tumor xenografts, MCF-7 cells were implanted s.c. in female athymic nude mice. When tumors reached ~200 mg, mice (n = 5/group) were randomized to receive either vehicle control or AF (60 mg/kg, i.p.) daily for 4 days. As shown in Fig. 6A, AF exerted a cytostatic effect on tumor growth (P < 0.01), relative to vehicle-treated mice. Notably, AF inhibited HIF-1α protein accumulation in tumor lysates by 90% (P < 0.005), relative to vehicle-treated mice (Fig. 6B) and mRNA expression of the HIF-1 target genes VEGF, CA9, and PDK-1 by ~70% (P < 0.05; Fig. 6C), showing that AF inhibits HIF-1α expression and activity in MCF-7 xenografts.

Because of the potentially confounding effects of tumor size on HIF-1α expression, we also conducted experiments in which MCF-7 tumor–bearing animals were only treated for 2 days with either vehicle control or AF (120 mg/kg, i.p.). As shown in Supplementary Fig. S6, AF did not affect tumor growth under these conditions, yet it significantly inhibited HIF-1α protein accumulation and VEGF mRNA expression (Supplementary Fig. S6B and C, respectively), suggesting that inhibition of HIF-1α and HIF-1 target genes by AF is independent from its cytostatic/cytotoxic activity.

**Discussion**

HIF-1 has emerged over the last several years as an attractive target for the development of novel cancer therapeutics. We and others have identified small-molecule inhibitors of HIF-1α that act by distinct mechanisms of action, including, but not limited to, inhibition of HIF-1α translation (31, 34, 37, 38), inhibition of HIF-1 DNA binding (39) or transcriptional activity (40–42), inhibition of protein–protein interaction (43), increased protein degradation (44, 45), or inhibition of mitochondrial respiration (46). Several HIF-1 inhibitors identified at the NCI share the property of inducing DNA damage, including topotecan (30) and NSC 644221 (34), although DNA damage and HIF-1 inhibition seem to be concomitant but independent events.

AF induces DNA damage and acts as a ligand of AhR. AF exerts antiproliferative activity in a fairly limited number of human cancer cell lines, and activation of AhR seems to be required for its conversion to DNA-damaging species, at least in part, by transcriptional activation of CYP1A1 and SULT1A1, two XRE target genes (18). In this article, we provide evidence that AF inhibits HIF-1α expression in MCF-7 cells in an AhR-independent fashion, as indicated by the following results: (a) AF inhibited HIF-1α protein accumulation in MCF-7 and AhR100 cells, in which the AhR pathway is functionally impaired; (b) AF did not inhibit HIF-1α in the resistant MDA-MB-231 breast cancer cells, yet it did in MDA/SULT1A1, MDA-MB-231 cells transfected with SULT1A1; and (c) siRNA targeting SULT1A1 significantly blocked the ability of AF to inhibit HIF-1α in MCF-7 cells (Supplementary Fig. S2). Along with the ability of AF to exert antiproliferative activity in MCF-7 and MDA/SULT1A1, but not in AhR100 or MDA-MB-231, these results suggest that (a) inhibition of HIF-1α by AF is independent from a functional AhR pathway, and (b) there is no correlation between cytotoxicity and HIF-1α inhibition. Indeed, AF was completely ineffective in inducing antiproliferative effects in AhR100 cells, yet it was able to inhibit HIF-1α expression. In addition, AF induced significantly lower levels of γH2AX, a marker of DNA damage, in AhR100 and MDA-MB-231 cells, which did not correlate with its ability to inhibit HIF-1α. These results not only show a dissociation between cytotoxicity and HIF-1 inhibition, but they also raise the possibility that cancer cells found to be “resistant” to the cytotoxic effects of AF may be sensitive to HIF-1α inhibition.

A significant number of small-molecule inhibitors of HIF-1α identified thus far seem to affect HIF-1 translation (31, 34, 37, 38), yet regulation of HIF-1α translation under hypoxic conditions is still poorly understood. We were then intrigued by the fact that AF also seemed to inhibit HIF-1α synthesis. Further experiments showed that AF inhibited HIF-1α mRNA expression by ~50%, under hypoxic, but not normoxic conditions. The magnitude of HIF-1α mRNA inhibition was unlikely to account for the almost complete inhibition of HIF-1α protein accumulation, raising the possibility that additional mechanisms were implicated. Using primers that specifically detect HIF-1α pre-mRNA, we discovered that hypoxia (in the absence or presence of AF) induced higher levels of transcript, relative to normoxic cells. The increased levels of HIF-1α pre-mRNA did not seem to correlate with the decreased expression of HIF-1α mRNA in the presence...
of AF. Notably, experiments conducted in the presence of actinomycin D showed that active transcription was required for the downregulation of HIF-1α mRNA expression by AF. The discrepancy between accumulation of HIF-1α pre-mRNA and decrease of HIF-1α mRNA levels might have suggested a potential effect of AF plus hypoxia on HIF-1α mRNA processing and/or maturation. However, results obtained in the presence of actinomycin-D argue against this conclusion and are consistent with transcriptional induction of either (a) a repressor, which in turn is responsible for inhibition of HIF-1α mRNA expression, or (b) noncoding RNA species, which may account for both inhibition of HIF-1α mRNA expression and translation. The latter possibility is consistent with (a) the mechanism of action of AF, which implicates protein nucleic acid complexes (33, 35), (b) the lack of correlation between magnitude of HIF-1α mRNA inhibition and inhibition of HIF-1α translation, and (c) the ability of topotecan, a DNA-damaging agent that also inhibits HIF-1α translation, to increase the levels of antisense transcripts of the HIF-1α genomic sequence (47). Several miRs have been recently identified that indeed target the 3′-untranslated region of HIF-1α mRNA, leading to the inhibition of HIF-1α protein levels (48–50). It is then plausible that the effects of DNA-damaging agents on HIF-1 protein translation may at least in part implicate noncoding RNA species that target HIF-1α mRNA expression and/or HIF-1α mRNA translation. Further experiments are required to characterize the spectrum of miR induced by agents that concomitantly inhibit HIF-1α and induce DNA damage to formally show the existence of a mechanistic link.

The proposed mechanism of HIF-1α inhibition by AF is conceivably associated with effects on other genes and pathways. However, AF did not inhibit NF-κB or AP-1 transcriptional activities and potently induced p21 mRNA and...
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank members of the Melillo’s laboratory, Yves Pommier, and Robert H. Shoemaker for the helpful discussion.

Grant Support

Federal funds from the NCI, NIH, under contract no. N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. This research was supported by the Developmental Therapeutics Program, DCTD, of the NCI, NIH.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 03/26/2010; revised 06/16/2010; accepted 06/30/2010; published OnlineFirst 08/24/2010.

References

28. Spink BC, Katz BH, Hussain MM, et al. SULT1A1 catalyzes...


Kaluz S, Kaluzova M, Stanbridge EJ. Proteasomal inhibition attenuates transcriptional activity of hypoxia-inducible factor 1 (HIF-1) via specific effect on the HIF-1α C-terminal activation domain. Mol Cell Biol 2006;26:5895–907.


Aminoflavone, a Ligand of the Aryl Hydrocarbon Receptor, Inhibits HIF-1 α Expression in an AhR-Independent Fashion

Erika Terzuoli, Maura Puppo, Annamaria Rapisarda, et al.

Cancer Res 2010;70:6837-6848. Published OnlineFirst August 24, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-1075

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/08/20/0008-5472.CAN-10-1075.DC1

Cited articles
This article cites 50 articles, 29 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/17/6837.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/70/17/6837.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/70/17/6837.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.