

Geldanamycin Induces Degradation of Hypoxia-inducible Factor 1 α Protein via the Proteasome Pathway in Prostate Cancer Cells¹

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Abstract

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor composed of α and β subunits. HIF-1 is critically involved in cellular responses to hypoxia, glycolysis, and angiogenesis. Here, we show that treatment of prostate cancer PC-3 and LNCaP cells with the benzoquinone ansamycin geldanamycin, an Hsp90-specific inhibitor, induced degradation of HIF-1 α protein in a dose- and time-dependent manner under both normoxia and hypoxia. This inhibition was also shown in other common cancer types tested. Rapid degradation of nuclear HIF-1 α protein levels was accompanied by respective inhibition in HIF-1 α functional transcription activity of VEGF. No difference between HIF-1 α mRNA levels before or after geldanamycin treatment was found. Moreover, [³⁵S]methionine pulse-chase analysis revealed that HIF-1 α protein half-life was markedly decreased in the presence of geldanamycin compared with that in control. The geldanamycin-induced degradation of HIF-1 α was reversed by proteasome inhibitors lactacystin and MG-132. We conclude that geldanamycin induces reduction of HIF-1 α levels and its downstream transcriptional activity by accelerating protein degradation independent of O₂ tension. Thus, benzoquinone ansamycin drugs and their derivatives, such as 17-allyl-aminogeldanamycin, are excellent candidates as small molecule drug inhibitors of HIF-1 overexpression in cancer cells.

Introduction

Hypoxia-inducible factor 1 (HIF-1) is an oxygen-regulated transcriptional activator that plays essential roles in tumor cell adaptation to hypoxia and angiogenesis (1). HIF-1 is overexpressed in common cancers and their metastases, including prostate, breast, colon, and lung cancers (2). HIF-1 is a heterodimeric protein composed of HIF-1 α and HIF-1 β subunits. HIF-1 α protein expression is induced in hypoxic cells and subjected to degradation in the presence of oxygen via ubiquitin-proteasomal pathway that is mediated by the pVHL³ (1). This process involves interaction between conserved subsequences within the oxygen-dependent degradation domains of HIF-1 α subunits and the β -domain of pVHL, with pVHL acting as the recognition element of a multicomponent E3 ubiquitin ligase. Recently, it was demonstrated that the interaction of the HIF-1 α with pVHL is regulated through oxygen-dependent prolyl hydroxylation within the HIF-1 α degradation domain (3–5).

HIF-1 drives transcription of many genes involved in tumor cell hypoxic adaptation and survival (1). HIF-1-regulated genes include the *VEGF*, *glucose transporters 1* and *3*, most of the glycolytic enzymes, and *erythropoietin*. These gene products are essential for high glycolytic rates of solid tumors (Warburg effect; Ref. 6). Overexpression of HIF-1 α is also associated with increased growth rate and metastatic potential of PCA, as well as with increased expression of HIF-1 α protein in the tumors (7). Recently, amplification of *HIF-1 α* gene was demonstrated in PC-3 cells (8). Overexpression of HIF-1 α in tumors makes it a potential therapeutic target in oncologic drug discovery. Inhibition of HIF-1 or genetic disruption can contribute not only to block tumor angiogenesis but also to block glycolytic metabolism and tumor cell growth, so that those tumor cells would be unable to respond to the hypoxic stimulus and progress (9, 10).

Newly synthesized HIF-1 α stably associates with the chaperone protein Hsp90 *in vitro* (11). Minet *et al.* reported that Hsp90 might regulate HIF-1 α activation in COS-7 cells (12). However, whether Hsp90 modulates HIF-1 α activity by stabilization of the protein or by another mechanism is not clear. We tested the hypothesis that pharmacological inactivation of Hsp90 protein could reduce HIF-1 α protein expression and, consequently, impair its transcriptional activity in PCA cells. The ansamycin antibiotic GA and its modified derivative 17-AAG bind to a conserved pocket in the Hsp90 protein and inhibit its function (13). Similar to GA, 17-AAG has antitumor activity in cell culture and animal xenograft models and is currently in clinical trial (14). We report that GA induces degradation of HIF-1 α protein through the proteosomal pathway, and HIF-1 α is a clear downstream target of Hsp90 inhibitors.

Materials and Methods

Cell Lines and Culture Conditions. The human PCA cell lines, PC-3 and LNCaP, were maintained with RPMI 1640 supplemented with 10% FBS at 37°C and 5% CO₂. The cells were subjected to hypoxia (1% O₂) as described previously (15).

Reagents and Antibodies. GA, LCN, and MG-132 were purchased from Alexis Biochemicals (San Diego, CA) and dissolved in DMSO. Purified mouse monoclonal anti-HIF-1 α antibody (1:500) was obtained from BD Transduction Laboratories (Lexington, KY). Monoclonal antibodies against HIF-1 β (1:500) and Hsp90 (1:500) were from Novus Biologicals (Littleton, CO). Polyclonal human antibody to human TOPO-I (1:1000) was purchased from TopoGEN (Columbus, OH). Secondary monoclonal antibody was horseradish peroxidase-conjugated sheep antihuman immunoglobulin (1:5000) and purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Preparation of Nuclear Extracts and Immunoblot Assay. Cells were seeded in either 6-well or 100-mm cell-culture dishes and grown in complete medium containing 10% FBS until 70% confluence. The culture media were then aspirated, and the cells were washed with serum-free medium. The cells were incubated with different reagents or with vehicle (0.1% DMSO) in media containing 0% or 10% FBS. Nuclear extracts and immunoblot assays were prepared as described previously (15). Proteins were detected with anti-HIF-1 α and the membranes were then stripped and reprobed with anti-TOPO-I as loading control. Sixty μ g of protein were loaded in each lane.

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³The abbreviations used are: pVHL, von Hippel-Lindau protein; VEGF, vascular endothelial growth factor; PCA, prostate cancer; Hsp90, heat shock protein 90; GA, geldanamycin; 17-AAG, 17-allyl-aminogeldanamycin; FBS, fetal bovine serum; LCN, lactacystin; TOPO-I, topoisomerase I; RT, reverse transcription.

Transient Transfections and Reporter Gene Assay. The pBI-GL construct (pBI-GL V6L) containing six tandem copies of the VEGF hypoxia response element was described (16). PC-3 cells growing in medium with 10% FBS in 6-well culture dishes were transiently transfected in triplicate with 1 μ g (each well) pBI-GL V6L using GenePorter transfection reagent (Gene Therapy Sys, Inc., San Diego, CA; Ref. 16). After 5 h of transfection, the cells were allowed to recover overnight. The cells were then washed twice with PBS and replenished with medium containing 10% FBS and vehicle or reagent. Duplicate sets of transfected cell-culture dishes were then separated and incubated either under normoxic or hypoxic conditions for 16 h. Luciferase enzymatic activity was measured with commercial kit TROPIX (Bedford, MA) using a BMG Labtechnologies LUMistar Galaxy luminometer and following the manufacturer's instructions. Arbitrary Luciferase activity units were normalized to the amount of protein in each assay point. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL).

HIF-1 α Half-Life Determination. PC-3 cells grown in 6-well plates were incubated in methionine- and cysteine-free as well as serum-free RPMI 1640 for 45 min. [³⁵S]methionine (ICN Biomedicals, Inc., Irvine, CA; final concentration, 120 μ Ci/well) was then added, and cells were incubated for 1.5 h. The radioactive medium was then removed, and cells were recultured in complete medium with 0.1% DMSO or 10 μ M GA for various times. Subsequently, the cells were washed twice with ice-cold PBS, lysed in 500 μ l of lysis buffer [20 mM sodium-HEPES (pH 7.5), 0.5% NP40, 0.1 M NaCl, 2 mM EDTA, 10% glycerol, and 2 mM DTT supplemented with protease and phosphatase inhibitors] and subjected to immunoprecipitation using anti-HIF-1 α antibody and protein G-agarose beads (Pierce) according to the manufacturer's instructions.

Isolation and Analysis of RNA. Total RNA was isolated from PC-3 cells treated and untreated with GA using TRIzol Reagent (Life Technologies, Inc.). Total RNA (15 μ g) was subjected to Northern blotting using human HIF-1 α cDNA probe (593-bp *Hind*III/*Msp*I fragment), as described previously (7), or VEGF cDNA probe (full-length human VEGF-165). A one-step real-time RT-PCR technique was performed using a thermocycler with continuous fluorescence-monitoring capabilities (iCycler iQ Multi-Color Real Time PCR Detection System, Bio-Rad) and SYBR Green I (Molecular Probes, Eugene, OR) to analyze the kinetics of PCR product accumulation. A reaction mix provided in the Titan One Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN) included 0.3 μ g of total RNA and 0.4 μ M final concentration of both forward (5'-agacatcgcggggaccgattcaccatg-3') and reverse (5'-tcggctagttaggtacac-3') HIF-1 α -specific primers. The reaction involved an initial incubation to allow a RT step at 50°C for 30 min, followed by 40 cycles of 92°C denaturation, 62°C annealing, and 68°C extension.

Data Analysis. Each experiment was performed independently three times. Thus, experiments presented in the figures are representative of three or more different repetitions. Quantification of band densities was performed using the public domain NIH Image (version 1.61). Statistical analysis was performed using a one-way ANOVA test ($P < 0.05$ was considered statistically significant).

Results

GA Reduces HIF-1 α Protein Level in PCA Cells. To test whether inhibition of HIF-1 α activity by GA is caused by destabilization of HIF-1 α protein, PC-3 cells were treated with escalating concentrations of GA. As shown in Fig. 1, HIF-1 α protein expression was blocked by GA in the absence and presence of serum (10% FBS) in a dose dependent pattern under normoxia (Fig. 1A). The same pattern of inhibition was also seen under hypoxia (Fig. 1B). GA was more potent, in inducing significant degradation of HIF-1 α protein in the absence than presence of serum (about 100-fold) under normoxia (Fig. 1A) and to a lesser extent (about 10-fold) under hypoxia (Fig. 1B). Densitometry measurements of the ratio of HIF-1 α /TOPO-I bands at each GA concentration point from 3 independent experiments revealed IC₅₀ values of 60 \pm 18 nM (mean \pm SD) and 4.61 \pm 2.26 μ M ($P = 0.025$) in the absence and presence of serum under normoxia, respectively. GA also decreased HIF-1 α protein in LNCaP cells under hypoxic conditions (Fig. 1C). To confirm that GA-induced reduction of HIF-1 α protein levels is not a consequent event of a lower degree of expression, we measured HIF-1 α mRNA levels using

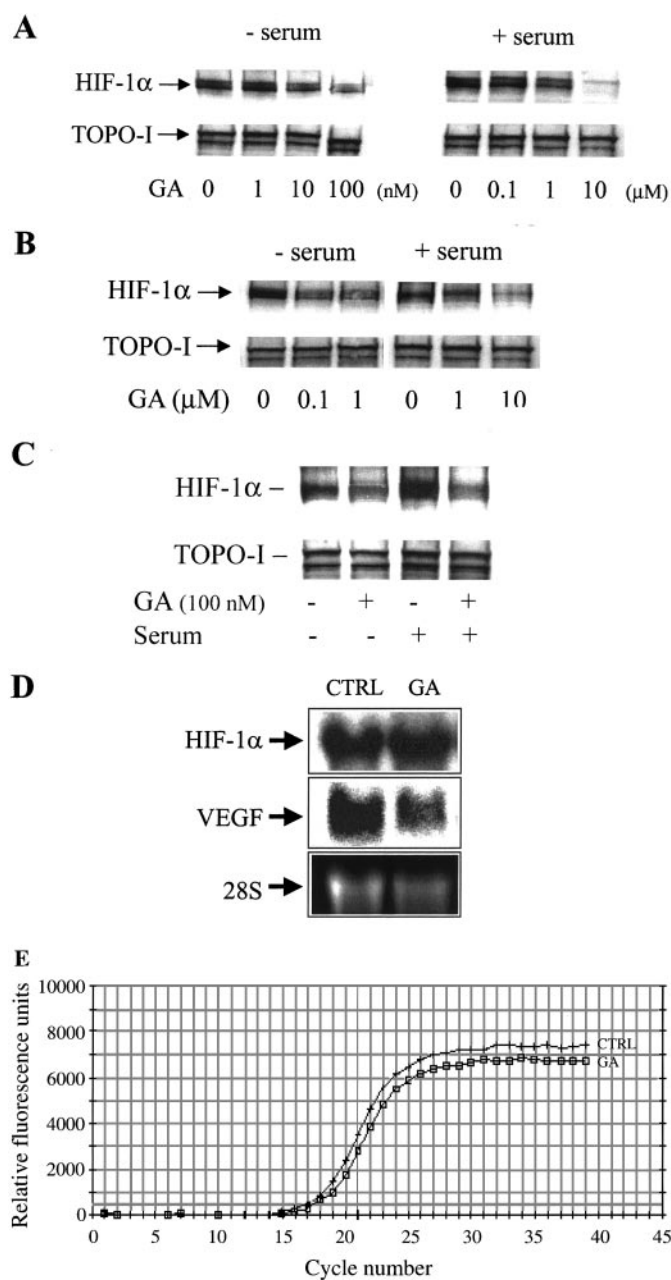


Fig. 1. GA treatment causes a decrease in HIF-1 α protein level in PCA cells. PC-3 (A and B) or LNCaP (C) cells were treated with increasing concentrations of GA in the absence or presence of serum for 4 h under normoxic (A) or hypoxic (B and C) conditions, and then harvested; and nuclear extracts were prepared for Western blotting. Northern blot analysis for HIF-1 α and VEGF mRNA (D) and real-time RT-PCR analysis for HIF-1 α mRNA (E) in PC-3 cells treated either with vehicle only or with 10 μ M GA for 4 h under normoxia.

Northern blot and real time RT-PCR analyses (Fig. 1 D & E). There was no difference in HIF-1 α mRNA levels before or after GA treatment (Fig. 1 D & E) whereas VEGF mRNA levels were decreased after GA treatment (Fig. 1D).

GA-induced HIF-1 α Protein Degradation Is Time Dependent. GA decreases HIF-1 α protein expression in PC-3 within 2 h in the absence and presence of serum (Fig. 2A). This inhibition lasted for 4 h but, surprisingly, was then reversed by the 8-h time point (Fig. 2A). To test whether this reverse of inhibition was caused by drug inactivation, cells were incubated in the presence of GA for a continuous 8 h or for 4 h, after which the medium containing GA was replaced with fresh GA-containing medium. Indeed, refreshing the medium after 4 h

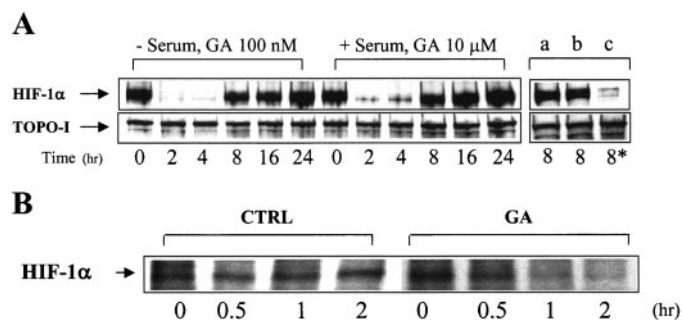


Fig. 2. GA treatment induces HIF-1 α protein degradation in a time-dependent manner and shortens its half-life. *A*, PC-3 cells were treated with GA in the absence or presence of serum for the indicated time periods under normoxia and then harvested for Western blotting. In Lanes *a-c*, cells were incubated without (*a*) or with 100 nM GA (*b* and *c*) in the absence of serum for 8 h, whereas the medium in Lane *c* was replaced by new medium containing fresh GA after 4 h. *B*, HIF-1 α protein stability was determined in the presence and absence (*CTRL*) of GA by [³⁵S]methionine pulse-chase analysis, as described under "Materials and Methods."

resulted in a complete inhibition of HIF-1 α protein up to 8 h of treatment (Fig. 2A, Lane *c*), whereas almost no inhibition was seen when GA was not refreshed or absent (Fig. 2A, Lanes *a* and *b*). To further confirm the hypothesis that GA destabilizes HIF-1 α , we measured HIF-1 α protein half-life by [³⁵S]methionine pulse-chase analysis in the presence and absence of GA (Fig. 2B). Exposure of cells to GA clearly resulted in a markedly decreased HIF-1 α protein half-life. In control cells, a true half-life could not be determined within 4 h (data not shown), whereas in GA-treated cells, the half-life of HIF-1 α protein was estimated to be less than 1 h (Fig. 2B).

GA-stimulated HIF-1 α Degradation Is Mediated by the Proteasome Pathway. Because HIF-1 α protein is degraded by the proteasome, and its stabilization under hypoxia occurs by escaping the proteasomal degradation, we examined whether HIF-1 α protein degradation by GA is mediated by the proteasome. PC-3 cells were treated with 10 μ M proteasome-selective inhibitor LCN (Fig. 3A). Compared with the control (Fig. 3A, Lane 1) LCN induced nuclear and cytosolic accumulation of HIF-1 α protein (Fig. 3A, Lane 3) whereas GA induced nuclear HIF-1 α protein degradation (Fig. 3A, Lane 2). Complete prevention from degradation was observed when the cells were simultaneously incubated with both GA and LCN (Fig. 3A, Lane 4). It is noteworthy to emphasize that the protein levels of HIF-1 β , Hsp90, and TOPO-I (loading control) proteins were unaffected by single or simultaneous treatment with GA and LCN (Fig. 3A). In addition, Northern blot and real-time RT-PCR analyses revealed that HIF-1 α mRNA levels were not changed after treatment with proteasome inhibitors (data not shown).

GA Inhibits HIF-1 α Transcriptional Activity. To determine the functional consequences of treating cells with GA on HIF-1 α transcriptional activity, we transiently transfected PC-3 cells with a Luciferase reporter gene construct (pBI-GL V6L) containing six tandem copies of the hypoxia response element derived from the human VEGF promoter (16). As shown in Fig. 3B, luciferase activity was significantly induced (15-fold) in response to hypoxia when cells were transfected with pBI-GL V6L, whereas no induction was seen with the empty vector (data not shown). HIF-1 α transcriptional activity was significantly inhibited by GA. To check whether this inhibition would be reversed by LCN as was shown on the protein level (Fig. 3A, Lane 3 and 4), transfected cells were treated with LCN alone or in combination with GA. Surprisingly, HIF-1 α transcriptional activity was inhibited in the presence of LCN (Fig. 3B). When added simultaneously with GA, the inhibition was far greater. To see whether this inhibition was exclusively LCN dependent or whether other proteasome inhibitors would have the same effect, we used MG-132. Similar

to LCN, MG-132 significantly abolished HIF-1 α transcriptional activity under normoxia as well as hypoxia (Fig. 3C) despite HIF-1 α protein accumulation and abrogation of GA-induced degradation (data not shown).

GA Induces Inhibition of HIF-1 α Protein in Various Cancer Cell Lines. To address whether GA-induced HIF-1 α protein destabilization is specific to PCA cells, we extended these studies to other cancer cell lines. As demonstrated in Fig. 4, GA induced various degrees of HIF-1 α protein degradation in the different cell lines tested. Densitometric quantification showed a decrease in HIF-1 α band after GA (10 μ M) treatment under hypoxia of 95, 75, 70, 80, 50, and 40% in prostate (PC-3), hepatoblastoma (HepG2), colonic ade-

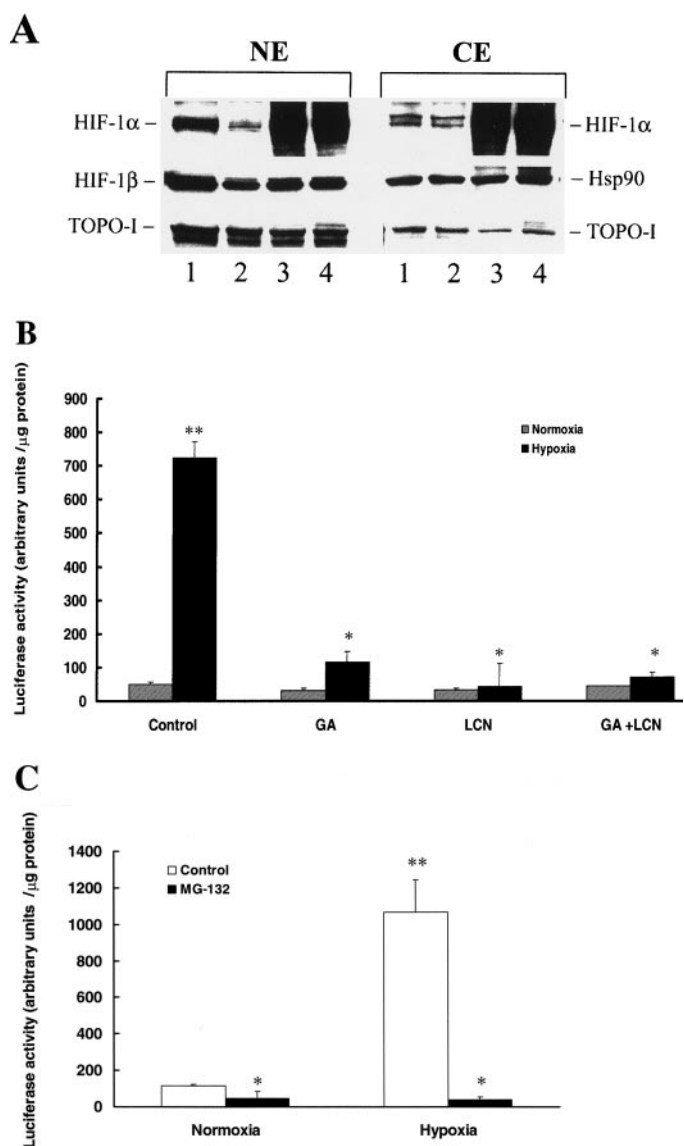


Fig. 3. GA-induced HIF-1 α protein degradation is mediated by the proteasome. *A*, PC-3 cells were treated with vehicle (Lane 1), 10 μ M GA (Lane 2), 10 μ M LCN (Lane 3), or 10 μ M GA and 10 μ M LCN (Lane 4) in the presence of serum, for 4 h under normoxic conditions. The cells were harvested and nuclear extracts (NE) and cytosolic extract (CE) were prepared for Western blotting with anti-HIF-1 α and reblotting with anti-HIF-1 β , anti-Hsp90, and anti-TOPO-I antibodies. *B*, PC-3 cells transiently transfected with pBI-GL V6L were treated with vehicle, 10 μ M GA, 10 μ M LCN, or 10 μ M GA and 10 μ M LCN under normoxic and hypoxic conditions. Luciferase reporter activity was measured in the cellular extract 16 h later. Luciferase activity represents arbitrary units per μ g protein in each assay point. Columns, means; bars, SD; *n* = 3; *, *P* < 0.001 compared with hypoxic control; **, *P* < 0.01 compared with normoxia. *C*, transfected PC-3 cells, as in *B*, were treated with vehicle or 5 μ M MG-132. Columns, means; bars, SD; *n* = 3; *, *P* < 0.05 compared with hypoxic control; **, *P* < 0.001 compared with normoxic control.

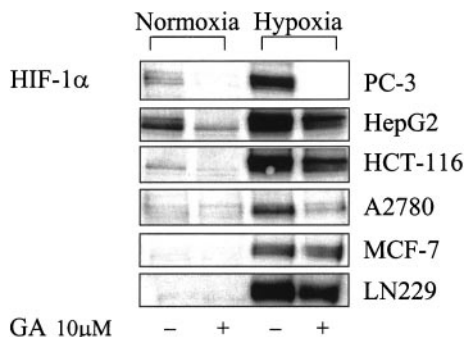


Fig. 4. GA stimulates HIF-1 α protein degradation in different cancer cells. The indicated cell lines were treated with vehicle or 10 μ M GA, each in its respective growing medium containing 10% FBS, under normoxic and hypoxic conditions for 4 h and then were harvested for Western blotting.

nocarcinoma (HCT-116), ovarian adenocarcinoma (A2780), MCF-7 breast, and glioma (LN229) cancer cell lines, respectively.

Discussion

This study shows that GA reduces nuclear HIF-1 α protein levels in prostate and other common cancer cells. Thus, GA and other Hsp90 ATP-binding site molecules can be predicted and combinatorially designed to have effects that include reducing expression of the HIF-1 “transcriptome” such as *VEGF* and other genes involved in angiogenesis and hypoxic adaptation. We further found that HIF-1 α protein degradation by GA is mediated via the ubiquitin-proteosomal pathway. It was previously shown that the overexpressed HIF-1 α protein interacts with the chaperone protein Hsp90 by immunoprecipitation (11). In addition, GA could inhibit HIF-1 transcriptional activity in COS-7 cells (12). GA is a benzoquinone ansamycin natural product of *Streptomyces hygroscopicus* subsp. *geldanus* that potentially inhibits the proliferation of a wide variety of tumor cell lines (17). Initial findings revealed that GA inhibits the activity of several different protein kinases, but subsequent studies revealed this to be an indirect effect of Hsp90 inhibition. GA specifically binds in the NH₂-terminal ATP binding site of Hsp90 and inhibits Hsp90-dependent ATPase activity. The GA-Hsp90 complexes cannot stabilize Hsp90 client proteins in a functional conformation, but result, instead, in their rapid destabilization and degradation, frequently mediated by the proteasome (17).

Our present results indicate that GA induced degradation of HIF-1 α protein in a dose- and time-dependent fashion with no effect on HIF-1 α mRNA levels (Figs. 1 and 2). It was previously shown that HIF-1 α protein could be stimulated by serum and other growth factors via the phosphoinositol-3-kinase pathway (18). GA-induced degradation was observed under no-serum and serum-supplemented conditions (Fig. 1), which may indicate that the GA effect could be dependent on the direct interaction between HIF-1 α and Hsp90 rather than other possible pathways involving phosphoinositol-3-kinase. Importantly, GA induced HIF-1 α protein degradation and inhibited HIF-1 α activity even under hypoxia. This demonstrates conclusively that Hsp90 is required to chaperone HIF-1 α protein under normoxia as well as hypoxia and is also essential for hypoxic HIF-1 activation. In addition, we found that GA-induced degradation is mediated via the proteasome pathway (Fig. 3A). Interestingly, blocking GA-induced HIF-1 α protein degradation with the proteasome inhibitors LCN and MG-132 failed to restore HIF-1 transcriptional activity (Fig. 3, B and C). In addition, these inhibitors, when used alone, dramatically inhibited HIF-1 activity in agreement with previous observations by Kallio *et al.* (19), who showed that the translocation of HIF-1 α from the cyto-

plasm to the nucleus was impaired in the presence of proteasome inhibitors. We believe that this inhibition is attributable to cytotoxic effects of the proteasome inhibitors because in our preparations HIF-1 α protein was shown to be accumulated, both in the nucleus as well as in the cytoplasm (Fig. 3A). However, even when lower doses of MG-132 (500 nM) were used that did not impair HIF-1 activity under normoxia, no restoration of the GA-induced inhibition of HIF-1 activity could be seen (data not shown). It is not clear whether this is because of the essential requirement of Hsp90 for folding HIF-1 α protein in the “correct” conformation or because of toxic effects of GA combined with MG-132.

Our data show that different degrees of degradation of HIF-1 α protein by GA occur in a wide spectrum of cancer cell lines, including prostate, hepatic, colonic, ovarian, brain, and breast (Fig. 4). This may be caused by a different dose dependency in these cells or to other possible cellular alterations that are not known at present.

Gan *et al.* (20) previously evaluated the antiproliferative and cytotoxic effects of GA in three human prostate xenograft tumors: the androgen-dependent CWR22 tumor and the androgen-resistant CWR22R and CWR91 tumors. The two androgen-resistant tumors were less sensitive than the androgen-dependent tumors to drug-induced antiproliferation but were about equally, or more, sensitive to drug-induced cytotoxicity (20). The inhibition of HIF-1 activity would, consequently, induce antiproliferation effects and is consistent with our observation that the androgen-sensitive LNCaP cells were more notably susceptible to GA-induced degradation of HIF-1 α protein than were the hormone-insensitive PC-3 cells (Fig. 1). Taken together, these data provide an important new rationale for the use of GA and its analogues as antineoplastic drugs in cells the survival of which depends in part on HIF-1 overexpression.

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