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Terpenoid Tetrahydroisoquinoline Alkaloids Emetine, Klugine, and Isocephaeline Inhibit the Activation of Hypoxia-Inducible Factor-1 in Breast Tumor Cells

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Klugine (**1**), isocephaeline (**2**), and emetine (**4**) inhibited hypoxia-inducible factor-1 (HIF-1) activation by hypoxia in T47D breast tumor cells (IC_{50} values 0.2, 1.1, and 0.11 μM, respectively). Compounds **1**, **2**, and **4** inhibited both hypoxia- and iron chelator-induced HIF-1 activation by blocking HIF-1α protein accumulation.

Within tumor masses, oxygen concentrations are highly variable, with oxygen partial pressures (pO_2) in many regions less than 5 mmHg (approximately 0.7% O_2).[1] Depending on the specific tumor type, the median pO_2 in tumors ranges from 5% to 34%, relative to those observed in the surrounding normal tissues.[1] This reduction in oxygen tension (hypoxia) activates both cellular survival and cell death programs.[2] In oncogenically transformed cells, hypoxia provides a physiological pressure and selects for the cells with diminished apoptotic potential.[3] Hypoxic tumor cells that have adapted to oxygen and nutrient deprivation are associated with a more aggressive phenotype and poor prognosis.[1,4]

The transcription factor that plays a critical role in hypoxia-induced gene expression is hypoxia-inducible factor-1 (HIF-1), a heterodimer of the oxygen-regulated HIF-1α and the constitutively expressed HIF-1β subunits.[5] Upon activation, HIF-1 induces the expression of genes that promote adaptation and survival under hypoxic conditions. Clinical studies indicate that HIF-1α overexpression correlates directly with advanced disease stages and poor prognosis in cancer patients.[6] The therapeutic potential of HIF-1 blockade for cancer treatment is supported by results from multiple studies employing animal models.[7] Intense research efforts are currently directed at the discovery and development of small molecule HIF-1 inhibitors for cancer.[5,8]

To discover novel natural products that inhibit HIF-1, thousands of plant, marine invertebrate, and microbial extracts were evaluated in a T47D human breast tumor cell-based reporter assay for HIF-1 inhibitory activity.[9] Three ethanol extracts prepared from different parts of the plant *Psychotria klugii* Standl. (Rubiaceae) all inhibited hypoxic activation of HIF-1 by greater than 90% at 5 μg mL^{-1}. Chromatographic fractions of *P. klugii* extracts and subsequently isolated pure compounds from the active fractions were further tested for HIF-1 inhibitory activity. The terpenoid tetrahydroisoquinoline alkaloids klugine (**1**), isocephaeline (**2**), and 7′-*O*-demethylisocephaeline (**3**) are analogues of the natural product emetine (**4**).[10] Emetine is a protein synthesis inhibitor that was evaluated in Phase II clinical studies as a potential chemotherapeutic agent for the treatment of solid tumors over 30 years ago.[11] The protein synthesis inhibitor cycloheximide (**5**) has been shown to inhibit HIF-1 activation.[12] Dose−response studies were performed to examine the effects of compounds **1**−**5** on HIF-1 activation by hypoxia (1% O_2) in a T47D cell-based dual luciferase reporter assay.[13] As shown in Figure 1A, compounds **1**, **2**, **4**, and **5** all inhibited HIF-1 activation by hypoxia (1% O_2, 16 h), while **3** was inactive at the concentrations tested (up to 30 μM). All four active compounds

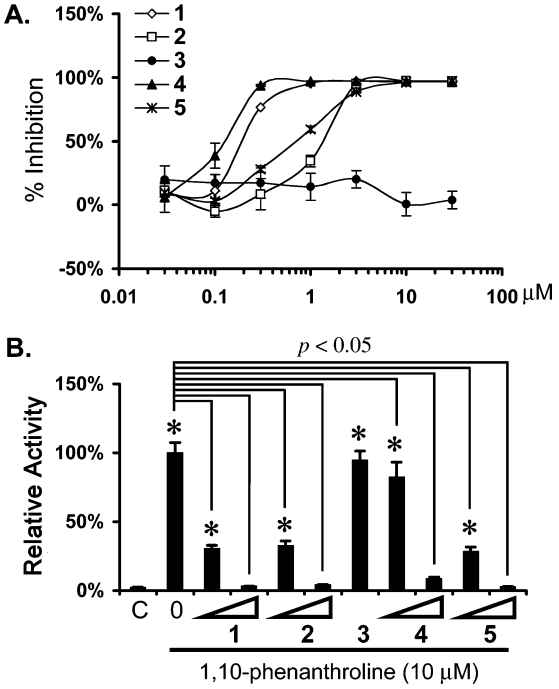

Figure 1. Dose−response of **1**−**5** on hypoxia-induced HIF-1 activation (A) and 1,10-phenanthroline-induced HIF-1 activation (B). (A) T47D cells that were transiently transfected with the pTK-HRE3-luc construct and an internal control pRL-TK were incubated with test compounds for 30 min before exposed to hypoxic conditions (1% O_2/94% N_2/5% CO_2) for 16 h. Following incubation, the cells were harvested and luciferase activities determined. Luciferase activity was normalized to that of the *Renilla* luciferase. The data are presented as percentage inhibition (as compared to the induced control) from one representative experiment performed in quadruplicate. Bars represent standard errors. (B) T47D cell-based dual luciferase reporter assay similar to that described for (A) except that 1,10-phenanthroline (10 μM) was used in place of the hypoxic conditions. An asterisk (*) indicates a significance of $p < 0.05$ when compared to the untreated control C. The compounds were tested at the following concentrations: **1** (0.2 and 1 μM); **2** (1.1 and 3 μM); **3** (10 μM); **4** (0.11 and 0.3 μM); and **5** (0.7 and 10 μM). All compounds except **3** exerted statistically significant ($p < 0.05$) inhibition of 1,10-phenanthroline-induced HIF-1 activation in T47D cells.

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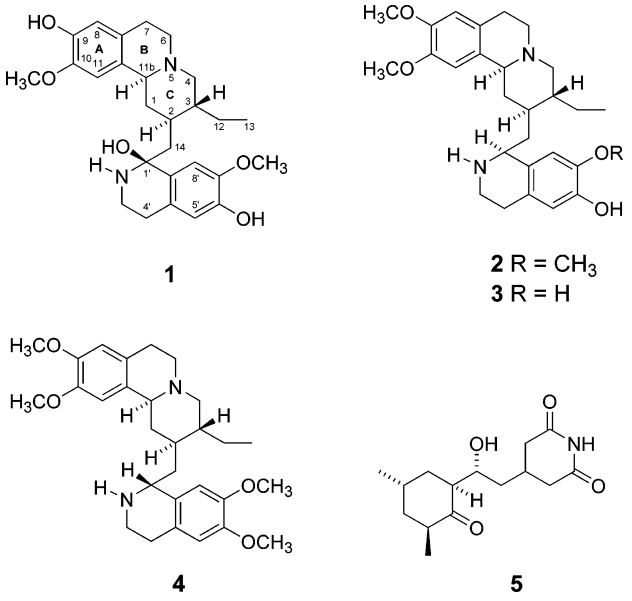
completely inhibited HIF-1 activation at 3 μM, with **4** being the most potent inhibitor (IC_{50} 0.11 μM, MIC 0.3 μM). Iron chelators and transition metals (such as cobalt and nickel) can activate HIF-1 and have been used as hypoxia mimetics.[9,14] Using a similar assay system, the effects of **1**−**5** on HIF-1 activation by the Fe^{2+} chelator 1,10-phenanthroline were evaluated to discern the specificity of HIF-1 inhibition. Each active compound (**1**, **2**, **4**, and **5**) was tested at two concentrations—IC_{50} and MIC (determined from Figure 1A)—and the inactive **3** at one single concentration (10 μM). At the higher concentration (MIC), compounds **1**, **2**, **4**, and **5** all completely inhibited HIF-1 activation by 1,10-phenanthroline, while **3** remained inactive (Figure 1B). One notable observation is that **4** inhibited 1,10-phenanthroline-induced HIF-1 activation to a lesser degree (18% inhibition) at the concentration (0.11 μM) that inhibits hypoxia-activated HIF-1 by 50%. These results suggest that these active compounds target a process (or processes) common to both hypoxia-induced and iron chelator-induced HIF-1 activation. Klugine (**1**) and emetine (**4**) have comparable HIF-1 inhibitory activities (Figure 1A), yet differ in both the degree of hydroxylation and pattern of *O*-methylation. Klugine (**1**) is essentially 1′-hydroxyl-9,6′-*O*-didemethylemetine. Apart from 6′-*O*-demethylation in **2**, compound **2** and emetine (**4**) differ only in that they are C-1′ epimers. Isocephaeline (**2**) has a *S*-configuration at C-1′, while emetine has a 1′*R*-configuration. However, **2** is approximately 10 times less potent than **4** at inhibiting hypoxia-induced HIF-1 (Figure 1A). Since klugine (**1**) is nearly as potent as **4**, it is likely the 1′*R*-configuration is a critical structural requirement for sub-micromolar HIF-1 inhibitory activity. This observation is mirrored by the significantly greater antiparasitic activity that has also been recently demonstrated for the 1′*R*-configured benzoquinolizidine alkaloid cephaeline, relative to its 1′*S*-epimer isocephaeline (**2**).[10] The fact that 7′-*O*-demethylisocephaeline (**3**) is essentially inactive (>27-fold less potent than **2**) suggests that the 7′-methoxyl group is also essential for HIF-1 inhibitory activity. Among the compounds tested, **3** is also the least cytotoxic to T47D breast tumor cells *in vitro* (Supporting Information).



Over 60 genes have been identified to be regulated by HIF-1.[5] One target gene that is induced by HIF-1 in most cell types is vascular endothelial growth factor (VEGF). As a potent angiogenic factor, VEGF functions at multiple stages of the angiogenic process. Currently, inhibition of

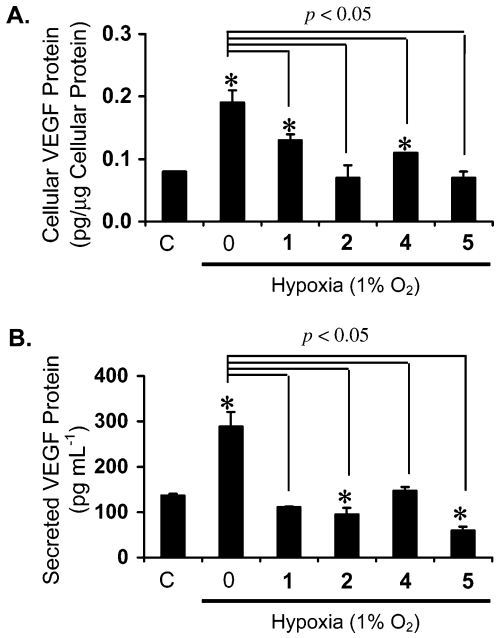


Figure 2. Inhibition of hypoxia-induced VEGF proteins in T47D cells. (A) VEGF protein levels in the cell lysates were determined by ELISA and normalized to the amount of cellular protein. Data shown are from one representative experiment performed in triplicate, and the bars represent standard errors. An asterisk (*) indicates a significance of $p < 0.05$ when compared to the untreated control C. The compounds were tested at the following concentrations: **1** (1 μM); **2** (3 μM); **4** (0.3 μM); and **5** (10 μM). All compounds exhibited statistically significant ($p < 0.05$) inhibition of VEGF protein induction by hypoxia. (B) Secreted VEGF protein levels in the conditioned media were determined by ELISA. Compound treatments and data presentation are as described for (A).

VEGF is under intense clinical evaluation as a new non-cytotoxic strategy for the treatment of solid tumors.[15] Hypoxia is a major tumor microenvironmental factor that induces VEGF expression.[15] The compounds found to inhibit HIF-1 activation were evaluated for their ability to inhibit hypoxic induction of VEGF (Figure 2). Hypoxic treatment (1% O_2, 16 h) increased both cellular and secreted VEGF protein production in T47D cells. When tested at the concentration equal to that of the MIC for HIF-1 activation in the reporter assay (determined from Figure 1A), compounds **1**, **2**, **4**, and **5** all significantly reduced the hypoxia-induced increase in cellular and secreted VEGF protein levels (Figure 2). Compounds **2** and **5** completely blocked the hypoxia-induced increase in cellular VEGF protein to levels that were statistically indistinguishable from that observed for a normoxic control (Figure 2A). Under these same conditions, compounds **1** and **4** inhibited the induction by 56% and 69%, respectively (Figure 2A). These compounds produced a similar effect on secreted VEGF protein levels (Figure 2B). While compounds **1** and **4** completely blocked the hypoxic induction of secreted VEGF protein, not only did compounds **2** and **5** inhibit the hypoxic induction, these two compounds further reduced the secreted VEGF protein levels to a point significantly below that observed for the untreated T47D cells (69% and 43% of the normoxic control level, respectively). At these same MIC concentrations, none of the active compounds exerted any significant effect on T47D cell viability or proliferation under hypoxic conditions (Supporting Information).

In general, the availability and activity of the oxygen-regulated HIF-1α subunit determines HIF-1 activity.[5,8] Under normoxic conditions, the HIF-1α subunit is post-translationally modified and rapidly degraded by the proteasome. Under hypoxic conditions, the HIF-1α subunit

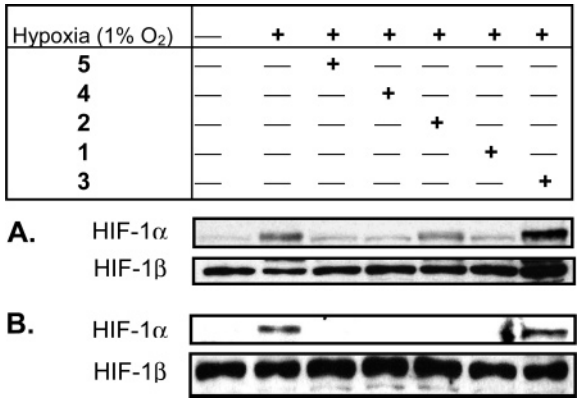


Figure 3. Effects of **1**−**5** on nuclear HIF-1α and HIF-1β proteins under hypoxic conditions. T47D cells were exposed to test compounds at 0.3 μM (A) and 10 μM (B) for 30 min prior to another 4 h incubation under hypoxic conditions (1% O_2/94% N_2/5% CO_2). The relative level of HIF-1α protein in each T47D nuclear extract sample was determined by Western blot using a monoclonal anti-HIF-1α antibody. The membrane was then stripped and the level of HIF-1β protein was determined by Western blot using a monoclonal anti-HIF-1β antibody.

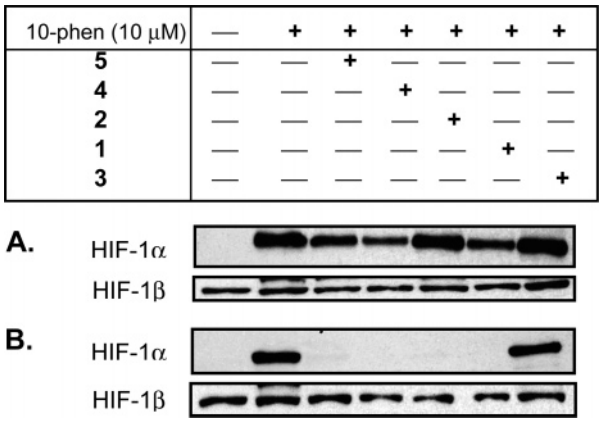


Figure 4. Effects of **1**−**5** on nuclear HIF-1α and HIF-1β proteins in the presence of 1,10-phenanthroline. Experimental procedures were as described for Figure 3 except that 1,10-phenanthroline (10 μM) was used to induce HIF-1α protein.

is stabilized and activated. The effects of compounds **1**−**5** on hypoxia-induced (1% O_2) accumulation of nuclear HIF-1α protein in T47D cells were examined (Figure 3). All compounds were tested at two concentrations: 0.3 and 10 μM. At the higher concentration, all of the compounds found to inhibit HIF-1 (**1**, **2**, **4**, and **5**) also blocked hypoxia-induced nuclear HIF-1α protein accumulation (Figure 3B). At the lower concentration, inhibition was only observed in the presence of **1**, **4**, and **5** (Figure 3A). The inactive analogue **3** did not exert any effect on the induction of nuclear HIF-1α protein. Under experimental conditions, none of the compounds affected the levels of the constitutively expressed HIF-1β protein in the nucleus. A similar study was conducted to examine the effects of compounds **1**−**5** on iron chelator-induced HIF-1α protein accumulation (Figure 4). At the higher concentration, compounds **1**, **2**, **4**, and **5** all blocked the induction of nuclear HIF-1α protein by 1,10-phenanthroline (10 μM, Figure 4B). At the lower concentration, only **1**, **4**, and **5** inhibited HIF-1α protein accumulation (Figure 4A). Compound **3** did not affect nuclear HIF-1α protein induction at either concentration.

Compounds **4** and **5** have both been demonstrated to inhibit protein synthesis at the aminoacyl transfer level.[16] However, the effects of **1**−**3** on protein synthesis have not been reported. One possible mechanism of action for these HIF-1 inhibitors (**1**, **2**, **4**, and **5**) is that they may inhibit *de novo* protein synthesis that is required for HIF-1α

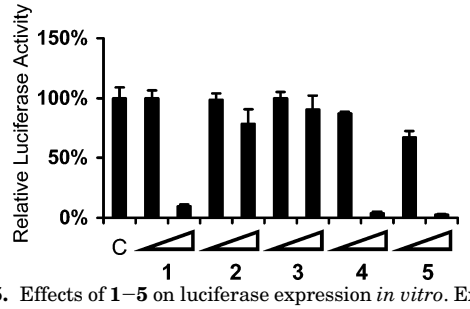


Figure 5. Effects of **1**−**5** on luciferase expression *in vitro*. Expression of luciferase from the Luciferase T7 Control DNA construct *in vitro* was carried out using the TNT T7 coupled reticulocyte lysate system. Luciferase activity was presented as percentage of the control "C." All compounds were tested at two concentrations: 0.3 and 10 μM. Data shown are averages obtained by determining the luciferase activities of three separate aliquots from each reaction mixture. Bars represent standard deviations. Similar results were obtained in a separate experiment.

protein induction. The effects of **1**−**5** on luciferase expression from a control construct (Luciferase T7 Control DNA, Promega) were examined *in vitro* (TNT T7 Coupled Reticulocyte Lysate System, Promega). In this assay, the level of luciferase expression is reflected by the relative luciferase enzyme activity. At the higher concentration tested (10 μM), compounds **1**, **4**, and **5** all inhibited luciferase expression (Figure 5). At the lower concentration (0.3 μM), neither **1** nor **4** affected luciferase expression in this coupled *in vitro* transcription/*in vitro* translation system. This is somewhat remarkable, since both **1** and **4** do inhibit HIF-1 activation at this concentration (0.3 μM). Compound **2** did not inhibit luciferase expression in this *in vitro* system (Figure 5). This is not entirely unexpected since the retention of chirality has been determined to be essential for the inhibition of protein synthesis in experiments with (−)-emetine and isoemetine.[17] One explanation may be that **1** functions in a fashion similar to **4** and **5** to block nuclear HIF-1α protein accumulation, while **2** may affect a yet to be identified target/pathway. As anticipated, the inactive compound **3** did not exert any effect on luciferase expression in this *in vitro* system.

Emetine (**4**) is 10 times more potent than cycloheximide (**5**) at inhibiting hypoxia-induced HIF-1 activation (Figure 1A). However, a recent study conducted in HeLa cells revealed that **5** is actually a more potent inhibitor of protein synthesis than **4** (IC_{50} 83 nM for **4** and 36 nM for **5**).[18] However, **4** was found to inhibit mitochondrial protein synthesis while **5** did not.[19] One possible scenario is that mitochondrial protein synthesis is critical for hypoxic signaling, rendering the hypoxic induction of HIF-1 significantly more sensitive to mitochondria-specific protein synthesis inhibitors. The fact that **4** is a protein synthesis inhibitor raises the possibility that the inhibitory effects of **4** on HIF-1 and its target genes (i.e., VEGF) is simply due to the inhibition of protein synthesis in general. The effect of **4** on the expression of secreted VEGF proteins in the absence of inducers (hypoxia or 1,10-phenanthroline) was examined in T47D cells. No inhibition was observed in the presence of **4** (control: 62.5 ± 4.6 pg mL^{-1}; **4** at 0.3 μM: 58.8 ± 7.6 pg mL^{-1}). This suggests that **4** most likely targets the process (or processes) specific to the transmission of hypoxic signals.

Klugine (**1**), isocephaeline (**2**), and cephaeline are the major alkaloids produced by *P. klugii*.[10] It is likely that **1**, **2**, and perhaps cephaeline function additively or synergistically and contribute to the potent HIF-1 inhibitory activity observed for extracts of *P. klugii*. Among these benzoquinolizidine alkaloids, emetine (**4**) is the most extensively studied. Emetine (**4**) is an active ingredient of ipecac

preparations (extracted from *Cephaelis ipecacuanha*) and has been mainly used as an emetic, as a treatment for amebiasis, and as an antibacterial or antiviral agent.[20] The antineoplastic potential of **4** was investigated in several clinical studies throughout the latter 20th century. As a single agent, **4** failed a Phase II clinical trial for solid tumor treatment.[11] No tumor regression was observed in patients receiving treatments. However, emetine (**4**) was reported to improve treatment outcome when used in combination with a chemotherapeutic agent (cyclophosphamide) for lung cancer.[21] In animal models, HIF-1 inhibition has been shown to enhance the outcomes of radiation and chemotherapeutic agents.[7e,22] It is possible that the HIF-1 inhibitory activity of **4** is responsible for augmenting the antitumor effects of cyclophosphamide observed in lung cancer patients.

Experimental Section

Compounds Used in Study. Compounds **1**−**3** were isolated from extracts of *Psychotria klugii*. Collection of the plant material, extraction, isolation, and structure elucidation of these specific compounds were previously reported.[10] An authentic sample of emetine (**4**) was kindly provided by Dr. S. Kuzii, University of the Pacific, Stockton, CA. Cycloheximide was purchased from Sigma, St. Louis, MO.

Cell Culture. Human breast carcinoma T47D cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM/F12 medium (JRH Biosciences, Lenexa, KS) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT) and 0.5% penicillin/streptomycin (equivalent to 50 units mL^{-1} and 50 $\mu g\ mL^{-1}$, respectively, Life Technologies, Inc., Gaithersburg, MD) at 37 °C in a humidified atmosphere (5% CO_2:95% air). Hypoxic conditions were achieved as previously described.[9]

T47D Cell-Based Reporter Assays. Dual luciferase reporter assay with the pTK-HRE3-luc reporter and pRL-TK construct (internal control, Promega, Madison, WI) was performed as described.[13] Reporter assay employing the pTK-HRE3-luc reporter was described previously.[9] In general, the cells were incubated with test compounds at 37 °C for 30 min before exposed to hypoxia (1% O_2) or iron chelator (1,10-phenanthroline at 10 μM).

ELISA Assay for Human VEGF Proteins. Plating of T47D cells, compound treatment, hypoxic exposure, preparation of cell lysates, and determination of VEGF protein level in the lysate by ELISA were performed as described.[13] The level of secreted VEGF proteins in the conditioned media was determined using a modified ELISA assay for human VEGF proteins (R&D Systems, Minneapolis, MN).[9]

Nuclear Extract Preparation and Western Blot Analysis for HIF-1α and HIF-1*β* Proteins. The procedures were the same as those described in detail.[9]

Neutral Red Assay for Cell Proliferation/Viability. Exponentially grown T47D cells were plated at the density of 30 000 cells per well in a volume of 100 μL of DMEM/F12 medium supplemented with 10% FCS (v/v) and 0.5% penicillin/streptomycin into 96-well tissue culture plate (Corning Inc., Corning, NY). Following 24 h incubation, test compounds were added in a volume of 100 μL of serum-free DMEM/F12 medium with 0.5% penicillin/streptomycin. After 30 min at 37 °C, the incubation continued for another 48 h under normoxic or hypoxic conditions. Cell proliferation/viability was determined using the Neutral Red method.[23] Briefly, the conditioned media were replaced with 100 μL per well DMEM/F12 medium that contains 10% FCS (v/v), 0.5% penicillin/streptomycin, and 0.17 $mg\ mL^{-1}$ Neutral Red (Sigma, St. Louis, MO). Neutral Red was prepared as a 1 $mg\ mL^{-1}$ stock solution in 1× PBS and filtered to sterilize. After 90 min incubation at 37 °C, the media were removed, the wells were washed once with 0.9% NaCl, and the Neutral Red was extracted from cells with 100 μL per well 0.04 N HCl in 2-propanol. The absorbances at 540 nm were measured on a microplate reader (BIO-TEK Instruments, Winooski, VT) with correction wavelength at 630 nm.

***In Vitro* Transcription/Translation Assay.** Coupled *in vitro* transcription/translation of the Luciferase T7 Control DNA construct (Promega, Madison, WI) was performed in a final volume of 12.5 μL using the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI) following manufacturer's instructions. One-fifth of the reaction mix was used for luciferase activity determination (Lucifearse Assay System, Promega, Madison, WI).

Statistical Analysis. Data were compared using ANOVA and post hoc analyses using Fisher's PLSD (StatView Software Version 5.01, SAS Institute Inc). Differences were considered significant when $p < 0.05$.

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Supporting Information Available: Figures showing T47D cell proliferation/viability in the presence of compounds **1**−**5** under normoxic and hypoxic conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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