Abietane Diterpenes from *Salvia miltiorrhiza* Inhibit the Activation of Hypoxia-Inducible Factor-1

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The hypoxia-inducible factor-1 (HIF-1) has been known to be correlated to the adaptation and proliferation of tumor cells; therefore HIF-1 has become an important target in the development of anticancer drugs. A phytochemical study of the $CHCl_3$-soluble fraction of *Salvia miltiorrhiza*, which strongly inhibited hypoxia-induced reporter gene expression, led to the isolation of 12 abietane-type diterpenes. Of these compounds, sibiriquinone A (**1**), sibiriquinone B (**2**), cryptotanshinone (**3**), and dihydrotanshinone I (**4**) potently inhibited hypoxia-induced luciferase expression with IC_{50} values of 0.34, 3.36, 1.58, and 2.05 μM on AGS cells, a human gastric cancer cell line, and 0.28, 3.18, 1.36, and 2.29 μM on Hep3B cells, a human hepatocarcinoma cell line, respectively. Consistently, **1** and **4** dose-dependently suppressed the HIF-1α accumulation and **1** inhibited mRNA expression of vascular endothelial growth factor (VEGF) under hypoxia. These results suggest that the anticancer activity of tanshinones is likely at least in part associated with their inhibition of HIF-1 accumulation.

Mammalian tumor cells exhibit many adaptive responses to hypoxic conditions, such as the transcriptional activation of angiogenic vascular endothelial growth factor (VEGF), erythropoietin (EPO), and other proteins in order to enhance oxygen delivery to the cells.[1] This adaptation is mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1), a heterodimer protein comprising HIF-1α and HIF-1β. HIF-1α protein is degraded rapidly under normoxic conditions and stabilized under hypoxic conditions, while HIF-1β protein is constitutively expressed.[2,3] Under hypoxia, HIF-1 binds to the hypoxia response element (HRE) to activate transcription of target genes, and the overexpression of the oxygen-regulated HIF-1α causes the proliferation of tumor cells and resistance against chemotherapy and radiotherapy in cancer treatment.[3,4] HIF-1 has been considered to be the center of most adaptation responses of cancer cells to hypoxia; therefore considerable efforts are underway to identify small-molecule HIF-1 inhibitors from chemical libraries and natural products.[5,6] At present, a number of HIF-1 inhibitors including natural antagonists have been in clinical trials as anticancer agents.[7,8]

The rhizome of *Salvia miltiorrhiza* Bunge (Labiatae) has been used in Oriental traditional medicine for treatment of menstrual disorders, hepatitis, and heart and cardiovascular diseases.[9,10] The major constituents of this species were abietane-type diterpene pigments, which showed various biological and pharmaceutical activities such as cytotoxicity, antitumor, antioxidant, anti-inflammatory, and NF-κB suppression.[10−12] Several alkaloids recently isolated from *S. miltiorrhiza* showed potent cytotoxicity against cervical epitheloid carcinoma, hepatocellular carcinoma, and ovarian adenocarcinoma cell lines.[13]

As a part of our search for HIF inhibitors from natural sources, the $CHCl_3$-soluble extract of *S. miltiorrhiza* strongly inhibited hypoxia-induced HIF-1 activation (70% inhibition at 4 $\mu g \cdot mL^{-1}$) in human gastric adenocarcinoma AGS cells. Phytochemical study of the $CHCl_3$-soluble extract led to the isolation of 12 known abietane diterpenes, of which sibiriquinone A (**1**), sibiriquinone B (**2**), cryptotanshinone (**3**), and dihydrotanshinone I (**4**) showed strong inhibitory activity against hypoxia-induced HRE-dependent reporter gene expression in AGS cells and in human hepatocellular carcinoma Hep3B cells, suggesting that these compounds may regulate the HIF-1 expression or stability. Compounds **1** and **4** were further evaluated for their effects on the hypoxia-activated HIF-1α accumulation and on the HIF-1 target gene, VEGF expression. The structure−activity relationship between these abietanes was also evaluated. The isolation of tanshinones from *S. miltiorrhiza* and an evaluation of their biological activity are the subject of this communication.

Results and Discussion

The structures of compounds **1**−**12** isolated from *S. miltiorrhiza* were characterized by spectroscopic methods and identified by comparison to reported data.[23,27−33] Of these compounds, sibiriquinone A (**1**), sibiriquinone B (**2**), and dihydroisotanshinone II (**8**) were isolated from this plant for the first time. The inhibitory effects of **1**−**12** on hypoxia-induced reporter gene expression were examined in an HRE-dependent luciferase expression assay in comparison with that of 17-desmethoxy-17-*N*,*N*-dimethylaminoethylaminogeldanamycin (17-DMAG) as a positive control.[3] The firefly luciferase activity was normalized by an internal control, *Renill*a luciferase activity (Figure 1). As shown in Table 1, most compounds except **8**, **10**, and **12** inhibited hypoxia-induced luciferase expression with IC_{50} values ranging from 0.34 to 15.1 and from 0.28 to 10.6 μM in AGS and Hep3B cells, respectively. Compound **1** was the most active and compounds **8**, **10**, and **12** were inactive at 20 μM, the highest concentration tested. The MTT assay in normoxic conditions showed that all the compounds had no significant cytotoxicity to both AGS and Hep3B cells at their effective concentrations for the inhibition of HRE-dependent luciferase expression except **7** and **9**, with a selectivity index (SI, ratio between LD_{50} value of cytotoxicity and IC_{50} value of HRE-dependent luciferase expression) of about 1.4 in AGS cells. Although selected compounds were slightly more toxic under hypoxia than under normoxia in AGS cells (Table 1), the SI values except **7** were acceptable. These indicated that the inhibitory activities of **1**−**6** and **11** in the HRE-dependent luciferase expression assay were not due to their cytotoxicity.

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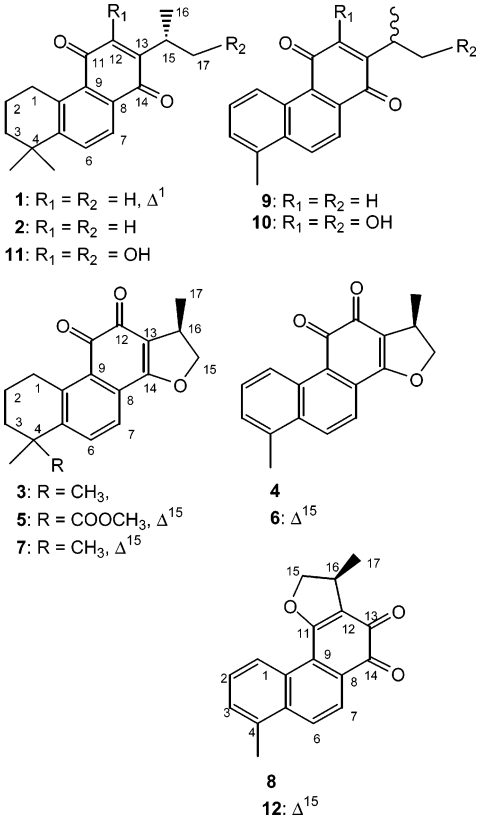

In the group of sibiriquinone A-like compounds (**1**, **2**, and **9**−**11**), the most active compound, **1**, possessing a double bond in the A ring, exhibited 10-fold stronger activity than **2**, having a saturated A ring. However, the presence of hydroxy groups (**10**, **11**) or aromatized A ring (**9**) decreased hypoxia-induced luciferase expression. The influence of the furo-*O*-naphthoquinone conjugation on the HIF-1 activity was also observed in the second group of tanshinone I-like compounds (**3**−**7**). The dihydrofurans (**3**, **4**) exhibited stronger activity than the furans (**5**−**7**), suggesting that saturation in the furan ring could play an important role in HIF-1 inhibition. This observation coincided with previous studies that revealed a critical role of the dihydrofuran ring in antiangiogenesis,[14] inhibition of acetylcholinesterase,[15] mast cell degranulation,[16] and diacylglycerol acyltransferase.[17] Furthermore, the A ring aromatization (**4**, **6**) also slightly decreased hypoxia-induced luciferase expression as compared with compounds **3** and **7**, having the saturated A ring. Weak inhibition was observed in the isotanshinone II-like group (**8**, **12**), possessing a different skeleton from the previous groups. These observations suggested that substitutions and quinone conjugations in the abietane skeleton influenced the inhibitory activity of the compounds.

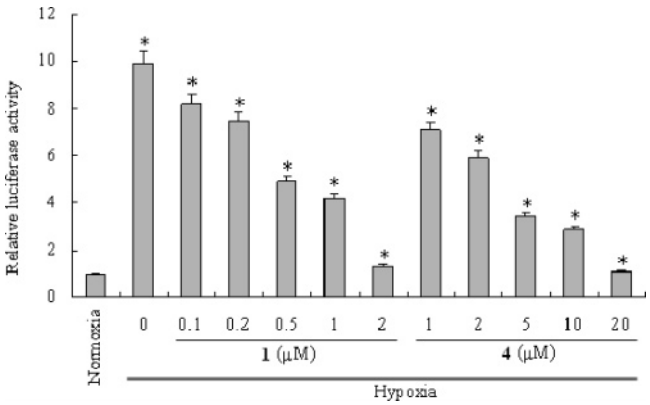

Figure 1. Relative luciferase activities of **1** and **4** were determined by the ratio of dual luciferase/*Renilla* luciferase activity. Hep3B cells were cotransfected with pGL3-HRE-luciferase vector and *Rluc* gene as the control. After 48 h, cells were treated with compounds in hypoxic conditions for 16 h, then cells were lysated and luciferase activities were measured. Bars represent standard deviations from three experiments. Asterisks (*) indicate a significance of $p < 0.05$ when compared to the untreated control.

To confirm whether these compounds inhibit the hypoxia-induced HIF-1α expression or stability, compounds **1** and **4**, representing the sibiriquinone A- and dihydrotanshinone I-type skeletons, respectively, were evaluated for their effect on hypoxia-induced HIF-1α accumulation by Western blot analysis in Hep3B cells. As shown in Figure 2A, both **1** and **4** blocked the HIF-1α accumulation in a dose-dependent manner with IC_{50} values of approximately 0.4 and 3.2 μM, respectively, which were comparable to those of reporter gene expression. The expression of several HIF-1 target genes, such as VEGF and EPO, is induced by hypoxia in most cell types. VEGF stimulates new blood vessel formation from existing vasculature, increasing tumor oxygenation and, ultimately, tumor growth and metastasis. This makes hypoxic tumors highly proangiogenic tumors with a highly aggressive phenotype.[1] Therefore, the effects of **1** on the hypoxia-induced VEGF mRNA expression in Hep3B cells was measured by RT-PCR analyses. The results showed that the hypoxia-induced VEGF mRNA expression was dose-dependently suppressed by **1** but not as effectively as it blocked the HIF-1α accumulation. This result led us to measure the VEGF protein concentration in the culture supernatant by ELISA. Consistently, the hypoxic induction of secreted VEGF protein was dose-dependently inhibited by **1** in an ELISA assay with an IC_{50} value of approximately 3.5 μM, which was also weaker than that observed in the HIF-1α accumulation (Figure 3). This difference may arise from the fact that VEGF mRNA transcription is also controlled by NF-κB-dependent transactivation through the κB response element in the promoter region independently of the oxygen tension in the culture condition.[18] A possible link between HIF-1 and NF-κB in the VEGF upregulation has also been shown to be an effect via the HIF-1/NF-κB/COX-2 pathway.[19] In addition to HIF-1 and NF-κB, other transcription factors such as AP-1, Sp1, and CREB have been identified within the VEGF promoters,[20,21] indicating that HIF-1 is not sufficient to induce the maximal level of VEGF under hypoxia. This suggested that **1** regulated VEGF expression in part through HIF-1, NF-κB, or other pathways. Potent antiangiogenesis activity of cryptotanshinone (**3**) in a previous study[14] may be related to the inhibition of both HIF-1 and NF-κB activation.[22] It is also known that HIF-1 controls genes responsible for the apoptosis resistance of tumor cells.[23,24] Consistently, compounds **4** and **6,** which were reported to induce apoptosis,[25,26] also exhibited strong inhibition of the HIF-1 activation in this study.

In conclusion, several abietane constituents were isolated from *S. miltiorrhiza* and exhibited inhibitory activity on hypoxia-induced HIF-1 expression or stability in AGS and Hep3B cells. Of these compounds, sibiriquinone A (**1**), sibiriquinone B (**2**), cryptotanshinone (**3**), and dihydrotanshinone I (**4**) were the most active, and compounds **1** and **4** suppressed HIF-1α accumulation. Furthermore, **1** suppressed VEGF mRNA expression. However, the molecular mechanisms of these compounds are still unclear. Further studies are needed to determine whether they inhibit HIF-1α expression at the translation or post-translation level, including its stability under hypoxic condition.

Experimental Section

General Experimental Procedures. Optical rotations were taken with a JASCO P-1020 digital polarimeter. UV spectra were recorded on a UV-1601 spectrometer. NMR experiments were performed on a Varian Unity Ionva-400 instrument. ESIMS were recorded on a Finnigan Navigator LC/MS/DS mass spectrometer. HPLC was performed using a Waters system with a 515 pump and a 2996 photodiode array detector.

Table 1. IC_{50} Values (μM) of **1**−**12** on Inhibition of HIF-1 Activation and Cell Viability

	AGS cell line		Hep3B cell line	
compound	HIF-1	cell viability	HIF-1	cell viability
sibiriquinone A (**1**)	0.34 ± 0.91	35.7 ± 0.16 (21.2 ± 1.05)[a]	0.28 ± 0.02	>50 (>50)
sibiriquinone B (**2**)	3.36 ± 1.62	>50 (>50)	3.18 ± 0.11	>50 (>50)
cryptotanshinone (**3**)	1.58 ± 1.16	13.5 ± 0.94 (10.2 ± 1.82)	1.36 ± 0.72	>30 (>30)
dihydrotanshinone I (**4**)	2.05 ± 0.58	>50 (47.7 ± 2.23)	2.29 ± 0.23	>50 (>50)
methyl tanshinonate (**5**)	7.48 ± 0.95	>50	7.34 ± 0.71	>50
tanshinone I (**6**)	12.5 ± 1.69	>50	8.51 ± 0.07	>50
tanshinone IIA (**7**)	7.53 ± 0.87	10.0 ± 0.09 (6.8 ± 0.41)	6.18 ± 1.48	>50 (>50)
dihydroisotanshinone II (**8**)	>20	>50	>20	>50
12-deoxytanshinquinone B (**9**)	15.1 ± 1.20	21.6 ± 2.30	10.6 ± 1.85	>50
danshenxinkun A (**10**)	>20	>50	>20	>50
neocryptotanshinone (**11**)	12.7 ± 0.85	>50	>20	>50
isotanshinone II (**12**)	>20	>50	>20	>50
17-DMAG	0.036 ± 0.24	16 ± 0.17	0.061 ± 0.83	>50

[a] Data in parentheses were obtained from hypoxic conditions.

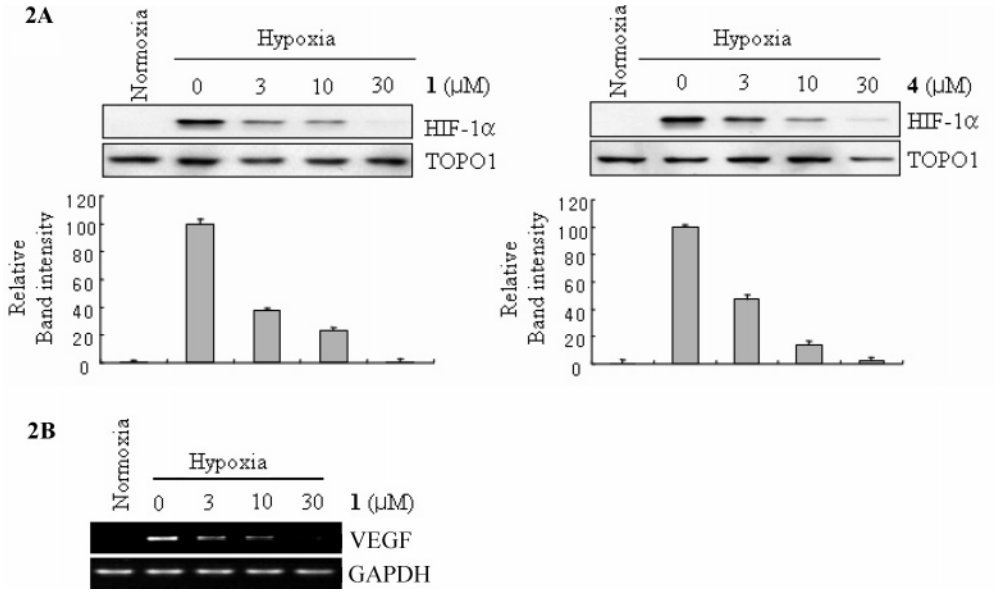

Figure 2. Effect of **1** and **4** on the accumulation of HIF-1α (A) and VEGF mRNA expressions (B) in Hep3B cells under hypoxic conditions. For A, Hep3B cells were exposed to various concentrations of **1** and **4** for 1 h and then incubated for 12 h under hypoxic conditions (1% O_2, 94% N_2, and 5% CO_2 at 37 °C). The relative levels of HIF-1α proteins were determined by Western blot using rabbit anti-HIF-1α antibodies. For B, Hep3B cells were exposed to various concentrations of **1** for 1 h and then incubated for 16 h under hypoxic conditions. Total RNA was isolated and VEGF mRNA was detected by a RT-PCR analysis as described in the Experimental Section. TOPOI and GADPH were used as internal controls to test loading and transfer efficiency.

Plant Material. A dry rhizome of *S. miltiorrhiza* (2.5 kg) was purchased from an herbal drug store in Daejeon, Korea, and identified by one of us (Y.H.K.). A voucher specimen (No. 050615) has been deposited in the Korean Research Institute of Biosciences and Biotechnology, Daejeon, Korea.

Extraction and Isolation. The dried and powdered rhizome of *S. miltiorrhiza* (2.5 kg) was extracted with MeOH (3 × 5 L) at room temperature. The extracts were combined, concentrated, and partitioned in $CHCl_3$−H_2O, and the organic layer was then evaporated *in vacuo* to give a brown solid (25 g), which was subjected to silica gel column chromatograpy eluted by a $CHCl_3$−MeOH gradient to afford four fractions, A1−A4. Fraction A2 was chromatographed on a silica gel column using *n*-hexane−EtOAc (10:1) as mobile phase to obtain **1** (21.0 mg), **2** (10.1 mg), and **6** (50.0 mg). Fraction A3 was eluted through a silica column with $CHCl_3$−MeOH (50:1), resulting in five subfractions, B1−B5. Fractions B1 and B2 were further purified by preparative TLC using *n*-hexane−EtOAc (6:1) to give **7** (5.6 mg) and **9** (3.4 mg), respectively. Preparative HPLC (YMC Pack Pro C18, 250 × 10 mm, 40% MeCN in H_2O, flow rate 10 mL·min^{-1}) of B3 afforded **3** (70 mg). Fraction B5 was fractionated by a Sephadex LH-20 column eluted by $CHCl_3$−MeOH (1:1) to afford **4** (5.8 mg), **5** (10.9 mg), and **8** (2.0 mg). Compounds **10** (10.0 mg), **11** (24.0 mg), and **12** (27.0 mg) were obtained from fraction A4 by repeated chromatography on a silica gel column with a mobile phase *n*-hexane−$CHCl_3$−MeOH gradient.

Sibiriquinone A (1): red powder; ESIMS *m/z* 281.4 $[M + H]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[23]

Sibiriquinone B (2): red powder; ESIMS *m/z* 283.4 $[M + H]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[27]

(−)-Cryptotanshinone (3): red powder; $[\alpha]_D^{25}$ −91.4 (*c* 0.1, $CHCl_3$); ESIMS *m/z* 297.4 $[M + H]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[28]

(−)-Dihydrotanshinone I (4): brown-red powder; $[\alpha]_D^{25}$ −82.2 (*c* 0.1, $CHCl_3$); ESIMS *m/z* 279.5 $[M + H]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[29]

(−)-Methyl tanshinonate (5): red powder; $[\alpha]_D^{25}$ −67.6 (*c* 0.1, $CHCl_3$); ESIMS *m/z* 339.4 $[M + H]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[30]

Tanshinone I (6): red powder; ESIMS *m/z* 277.0 $[M + H]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[31]

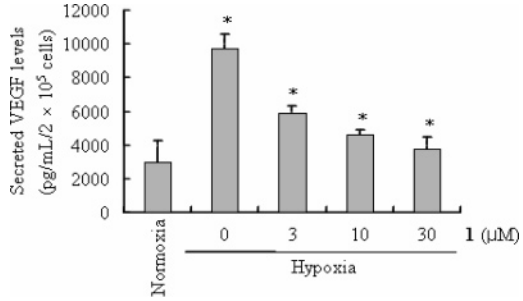

Figure 3. Effect of **1** on secreted VEGF levels. Hep3B cells (2×10^5 cells per well) were cultured with various concentrations of compound **1** for 16 h under normoxic or hypoxic conditions. The VEGF levels in culture supernatants were determined by using the DuoSet ELISA development kit. Bars represent standard deviations from five experiments. Asterisks (*) indicate a significance of $p < 0.05$ when compared to the untreated control.

Tanshinone IIA (7): red powder; ESIMS *m*/*z* 295.4 $[M + H]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[31]

(+)-Dihydroisotanshinone II (8): red powder; $[\alpha]_D^{25}$+30.5 (*c* 0.1, $CHCl_3$); ESIMS *m*/*z* 279.5 $[M + H]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[32]

12-Deoxytanshinquinone B (9): red powder; ESIMS *m*/*z* 263.5 $[M - H]^-$; ^1H and ^{13}C NMR data were in agreement with those previously published.[32]

(−)-Danshenxinkun A (10): red powder; $[\alpha]_D^{25}$ −102 (*c* 0.1, MeOH); ESIMS *m*/*z* 295.5 $[M - H]^-$; ^1H and ^{13}C NMR data were in agreement with those previously published.[29]

(−)-Neocryptotanshinone (11): red powder; $[\alpha]_D^{25}$ −40.7 (*c* 0.1, $CHCl_3$); ESIMS *m*/*z* 313.5 $[M - H]^-$; ^1H and ^{13}C NMR data were in agreement with those previously published.[33]

Isotanshinone II (12): red powder; ESIMS *m*/*z* 277.5 $[M + H]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[32]

Cell Culture. Human gastric adenocarcinoma AGS cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 (Invitrogen, Grand Island, NY), and human hepatocellular carcinoma Hep3B cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT), penicillin, and streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. The hypoxic culture was kept in a gas-controlled chamber (Thermo Electron Corp., Marietta, OH) maintained at 1% O_2, 94% N_2, and 5% CO_2 at 37 °C.

Reporter Assay. The ability of compounds to inhibit hypoxia-inducible factor-1 was determined by a reporter assay. Exponentially grown cells (5×10^6 cells) at 75−90% confluence were transiently cotransfected with the vectors for pGL3-HRE-luciferase plasmid,[34] which contains six copies of HREs derived from the human VEGF gene, and pRL-CMV (Promega, Madison, WI) using Lipofectamine Plus reagent according to the instructions of the manufacturer (Invitrogen). The transfected cells were plated at a density of 5×10^4 cells per well into 96-well plates. After 48 h incubation, the cells were treated with various concentrations of the test compounds and incubated for 16 h under hypoxic conditions (1% O_2, 94% N_2, and 5% CO_2). The luciferase assay was performed using a dual-luciferase reporter assay system according to the instructions of the manufacturer (Promega). Luciferase activity was determined in a Microlumat Plus luminometer (EG&G Berthold, Bad Wildbad, Germany) by injecting 100 μL of assay buffer containing luciferin and measuring light emission for 10 s. The results were normalized to the activity of *Renilla* luciferase expressed by a cotransfected *Rluc* gene under the control of a constitutive promoter. 17-DMAG (Calbiochem, La Jolla, CA) was used as a positive control.

Cytotoxicity Assay. Cell viability was evaluated by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method. Cells were seeded in a 96-well plate at 5×10^4 cells per well and incubated for 3 h at 37 °C. Then cells were treated with various concentrations of test compounds under normoxic or hypoxic conditions. After 24 h incubation, 10 μL of 5 mg·mL^{-1} MTT was added to each well and incubated for another 4 h. After removing of supernatant, formazan crystals were dissolved in 100 μL of DMSO, and the OD values were measured at 570 nm with a microplate reader.

Western Blot Analysis. Exponentially grown Hep3B cells were plated at a density of 1.5×10^5 cells per cm^2 growth area and incubated at 37 °C overnight. Various concentrations of test compounds were added and incubated for 1 h and then further incubated for 12 h under hypoxic conditions. At the end of incubation, the cells were harvested by scraping, followed by centrifugation at 1000*g* for 5 min at 4 °C, and washed twice with ice-cold phosphate-buffered saline. To analyze the level of HIF-1α proteins, total cell lysates were prepared from cells using a lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg·mL^{-1} leupeptin, 150 mM NaCl). Total cell lysates (50 μg) were separated by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a Hybond-C membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS) at room temperature for 1 h and then incubated overnight at 4 °C with an anti-HIF-1α rabbit antibody (1:2000 dilution in 5% nonfat milk in TTBS; R&D Systems, Minneapolis, MN). Horseradish peroxidase-conjugated anti-rabbit antiserum (Amersham Biosciences, Piscataway, NJ) was used as a secondary antibody (1:2500 dilution in 5% nonfat milk in TTBS, 2 h incubation), and the antigen−antibody complexes were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Bioscience, Piscataway, NJ). Protein loading was controlled by probing the membranes for TOPO I protein with an anti-TOPO I antibody (1:500 dilution in 5% nonfat milk in TTBS; Santa Cruz Biotechnology).

RT-PCR Analysis. After Hep3B cells were pretreated with various concentrations of compound **1** for 1 h, the cells were further incubated for 16 h under hypoxic conditions. Total RNA from cells was obtained using an RNA mini kit (Qiagen, Valencia, CA). Total RNA (2 μg) was used to perform reverse transcription-PCR (RT-PCR) using an RT-PCR kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The PCR primers for VEGF were 5′-GCTCTACCTCCAC-CATGCCAA-3′ (sense) and 5′-TGGAAGATGTCCACCAGGGTC-3′ (antisense). The oligonucleotide sequences of the reaction products were confirmed by sequencing.

VEGF ELISA. Hep3B cells were plated in a 96-well plate at a density of 2×10^5 cells per well and treated with various concentrations of compound **1** for 16 h under normoxic or hypoxic conditions. The VEGF levels in the culture supernatant were determined by ELISA using the DuoSet ELISA development kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Statistical Analysis. Each experiment was performed at least three times, and representative data are shown. Data in the table are given as mean values ± standard deviation from three separate experiments. Means were checked for statistical differences by using the Student's *t*-test with error probabilities of $p < 0.05$. Band intensity was analyzed by scanning densitometry (TINA software, version 2.10; Raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany).

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