An Isoaurone and Other Constituents from *Trichosanthes kirilowii* Seeds Inhibit Hypoxia-Inducible Factor-1 and Nuclear Factor-κB

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Hypoxia-inducible factor-1 and nuclear factor-κB have become important targets in cancer treatment due to their critical role in the regulation of genes involved in tumorigenesis. Bioassay-guided fractionation of the methanol extract of *Trichosanthes kirilowii* seeds led to the isolation of a naturally rare isoaurone, 4′,6-dihydroxy-4-methoxyisoaurone (**1**), together with three known compounds, cucurbitacin B (**2**), 6-(3-hydroxy-4-methoxystyryl)-4-methoxy-2*H*-pyran-2-one (**3**), and blumenol A (**4**). All compounds inhibited HIF-1 and NF-κB activities in reporter assays. Compounds **1**−**3** potently inhibited HIF-1α accumulation and VEGF secretion under hypoxic condition. These results suggest that the tumor cell growth inhibitory activity of *T. kirilowii* is likely associated with the inhibition of HIF-1 and NF-κB activities.

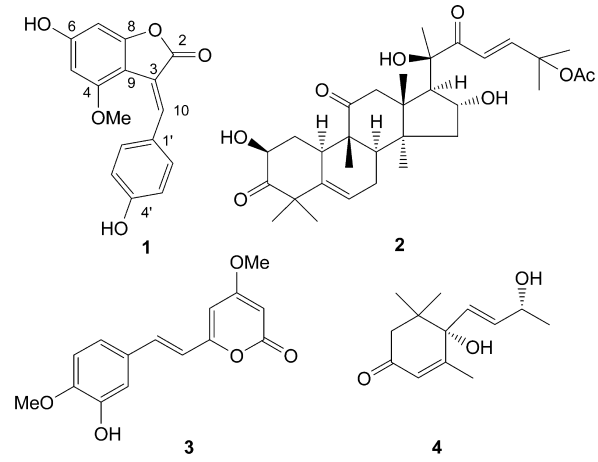
Hypoxia-inducible factor-1 (HIF-1) is one of the most important factors that play a critical role in controlling oxygen delivery and metabolic adaptation to hypoxic conditions. HIF-1 is a transcription factor composed of HIF-1α and HIF-1β subunits, with only HIF-1α being regulated by oxygen tension.[1] In response to physiological hypoxia, HIF-1α is rapidly stabilized and is localized in the nucleus, where the HIF-1 complex binds to a short DNA sequence, 5′-ACGTG-3′, known as the hypoxia-responsive element (HRE) within target genes and activates transcription. HIF-1α is overexpressed in many human cancers and has been associated with tumor aggressiveness, vascularity, and treatment failure.[2] All of these activities make the HIF-1 transcription factor an attractive target for the development of new cancer therapeutics.[3]

Nuclear factor-κB (NF-κB) is a family of Rel domain-containing proteins consisting of RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52).[4] The NF-κB activation results in the transcriptional regulation of various genes involved in multiple pathophysiological conditions such as cancer, arthritis, asthma, and inflammation.[5] Therefore, NF-κB and the signaling pathways that regulate its activity have become a focal point for drug discovery and development efforts.[6]

Trichosanthes kirilowii Maxim. (Cucurbitaceae) has been used for a long time in oriental traditional medicine. The seed, fruit peel, and root of the plant are widely used for treatment of cough, inflammation, diabetes, and obstipation.[7] A number of studies have been reported on the chemical constituents and biological activity of *T. kirilowii* including antitumor, anti-HIV, and antityrosinase.[8−11] Of note, the protein trichosanthin and the triterpene group of cucurbitacins showed potent antitumor activity.[12−14]

In our continuing search for inhibitors of HIF-1 and NF-κB from natural sources, a MeOH extract of the seed of *T. kirilowii* was found to potently inhibit HIF-1 and NF-κB activities in cell-based reporter assays. Phytochemical fractionation of the $CHCl_3$-soluble part of the MeOH extract led to the isolation of a new and three known compounds. The structure of the new isoaurone **1** was determined by spectroscopic methods, and the known compounds cucurbitacin B (**2**), 6-(3-hydroxy-4-methoxystyryl)-4-methoxy-2*H*-pyran-2-one (**3**), and blumenol A (**4**) were identified by comparing their physicochemical data with literature values.[14−16]

Compound **1** was obtained as a yellow solid, and its HRESIMS revealed an $[M + H]^+$ peak at *m*/*z* 285.2914, corresponding to the molecular formula $C_{16}H_{13}O_5$. The ^{1}H NMR spectrum of **1** showed the presence of an AA′BB′ aromatic system at δ_H 8.02 (2H, d, J = 8.8 Hz, H-2′,6′) and 6.82 (2H, d, J = 8.8 Hz, H-3′,5′), two *meta*-coupled aromatic protons at δ_H 6.18 (1H, d, J = 1.6 Hz) and 6.27 (1H, d, J = 1.6 Hz), and an isolated olefinic proton at δ_H 7.91 (1H, s, H-10). The ^{13}C NMR spectrum of **1** revealed the presence of eight quaternary carbon atoms, seven methines, and a methoxy group. The MS and 1D NMR evidence as well as the HMBC correlations of **1** (Figure 1) showing the deshielded H-10 (δ_H 7.91 and δ_C 141.3) coupled to C-2 (δ_C 168.5) suggested that the structure of **1** could be a dihydroxymethoxyisoaurone.[17,18] The AA′BB′ spin system in the ^{1}H NMR spectrum indicated one hydroxy group attached to C-4′ (δ_C 161.2). In the class of $C_6-C_3-C_6$ compounds possessing hydroxy substituents at C-5 and C-7, the chemical shift of C-6 (or C-5 in the case of **1**) appears at lower field than C-8 (or C-7 in the case of **1**);[19] therefore, the signals at δ_C 95.6 and 92.0 were assigned to C-5 and C-7, respectively. The HMQC spectrum indicated that the *meta*-coupled aromatic protons at δ_H 6.27 and 6.18 associated with C-5 and C-7, respectively. The HMBC spectrum also revealed the coupling from the O-methyl protons (δ_H 3.94) to the carbon atom at δ_C 158.0, which further showed correlation with H-5 (δ_H 6.27) but not with H-7 (δ_H 6.18). This indicated that the methoxy group was located at C-4 and the hydroxy group at C-6. The configuration of *E*- and *Z*-isoaurones is determined on the basis of the chemical shift of H-10, which is anisotropically and diamagnetically affected by the C-2 carbonyl group. It is known that the chemical shift of H-10 in *Z*-isoaurone



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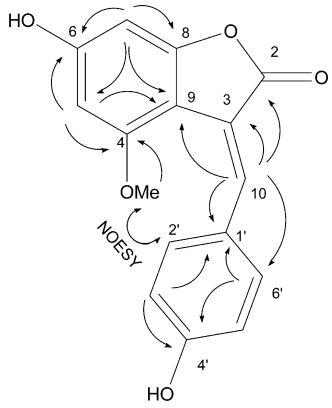

Figure 1. HMBC (→) and NOESY (↔) correlations of **1**.

Table 1. IC_{50} Values (μM)[a] of Compounds **1**−**4** on HIF-1, VEGF, and NF-κB Inhibition

compound	HIF-1	VEGF	NF-κB
1	0.69 ± 0.24	0.93 ± 0.38	0.56 ± 0.18
2	0.27 ± 0.09	0.31 ± 0.14	0.061 ± 0.02
3	0.81 ± 0.17	1.04 ± 0.22	0.40 ± 0.15
4	15.8 ± 1.20	26.2 ± 4.74	12.3 ± 2.37
17-DMAG[b]	0.057 ± 0.002	0.079 ± 0.01	
celastrol[c]			0.15 ± 0.47

[a] Data are means ± SD from three experiments. [b] Positive control for HIF-1 and VEGF activities. [c] Positive control for NF-κB activity.

resonates at higher field (7.4−7.5 ppm) than in the *E*-isomer (7.8−8.0 ppm).[18,20] Therefore, the H-10 chemical shift at δ_H 7.91 suggested that **1** is *E*-isoaurone. This conclusion was supported by the cross-peak between the methoxy group (δ_H 3.94) and H-2′, 6′ (δ_H 8.02) in a NOESY experiment. Thus, compound **1** was assigned as (*E*)-3-(4′-hydroxybenzylidene)-6-hydroxy-4-methoxybenzo-2(3*H*)-furanone, i.e., 4′,6-dihydroxy-4-methoxyisoaurone.

The effects of compounds **1**−**4** on HIF-1 activity were evaluated in an HRE-dependent reporter assay in human hepatocarcinoma Hep3B cells. Compounds **1**−**3** strongly inhibited HIF-1, while **4** was moderately active (Table 1). The MTT assay showed that the compounds had no significant toxicity to cells at their effective concentrations (data not shown), indicating that the inhibitory effects of these compounds were not due to cytotoxicity. Next, we evaluated the effect of **1**−**4** on HIF-1α levels by western blot analysis. Compounds **1**−**3** dose dependently inhibited hypoxia-induced HIF-1α accumulation in Hep3B cells, while **4** was weakly active (Figure 2). HIF-1 regulates the expression of several target genes such as vascular endothelial growth factor (VEGF). Therefore the effect of **1**−**4** on VEGF production was evaluated in the Hep3B cell culture media by ELISA assay. All compounds but **4** suppressed VEGF secretion with IC_{50} values comparable to those from the reporter assay (Table 1). The VEGF expression is also regulated by different transcriptional factors including NF-κB, and a link between HIF-1 and NF-κB in the VEGF regulation has been reported.[21] Therefore, the NF-κB reporter assay was carried out to examine whether the compounds inhibited NF-κB activity. The results indicated that all compounds but **2** inhibited NF-κB activation at doses comparable to those for the inhibition of HIF-1 and VEGF (Table 1). Interestingly, cucurbitacin B (**2**) inhibited NF-κB activation much more potently than celastrol, a known potent NF-κB inhibitor.[22]

Naturally occurring 2(3*H*)-benzofuranones (isoaurones) are rare, and only a few compounds have been reported.[17,23] The biosynthetic pathway to isoaurones is unclear; however, this class of compound could be derived from chalcones.[24,25] Several synthetic isoaurones showed strong cytotoxicity and inhibition of topoisomerases I and II.[18,26] In the present study, the isoaurone **1** was identified as a potent HIF-1 and NF-κB inhibitor. Cucurbitacin B (**2**) has been

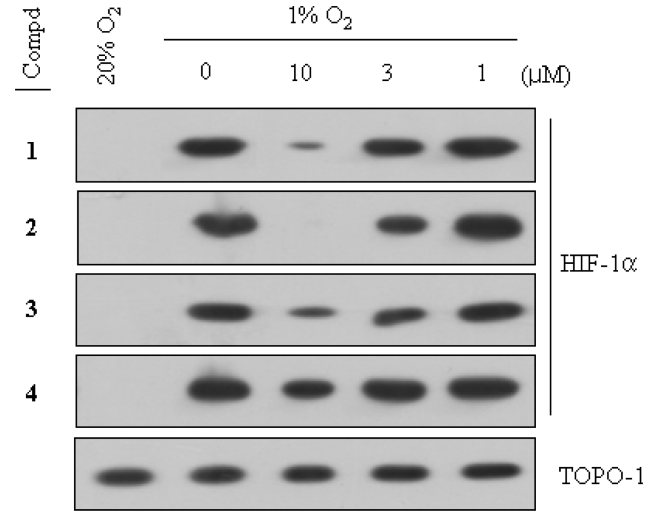

Figure 2. Effects of **1**−**4** on HIF-1α accumulation. Hep3B cells were exposed to various concentrations of compounds for 30 min and then incubated for 12 h under normoxic or hypoxic conditions. The relative levels of HIF-1α protein in nuclear extract were determined by western blot analysis using mouse monoclonal anti-HIF-1α antibody. Topo-1 was detected for loading control.

known as an active component of *T. kirilowii* in various biological activity investigations including tumor cell growth inhibitory activity.[27,28] Previous studies also showed that this compound induced apoptosis and suppressed tumor growth by targeting signal transducers and activators of transcription 3 (STAT3).[29] Our results suggest that the tumor cell growth inhibitory activity of this compound may be associated with the regulation of HIF-1 and NF-κB signaling. HIF-1, NF-κB, and STAT3 are important transcriptional factors that were found to play pivotal roles in the tumorigenesis, and their cross-talk relationship has been reported.[30] It has been demonstrated that STAT3 interacted with the C-terminal domain of HIF-1α and stabilized HIF-1α from ubiquitination-dependent degradation by inhibition of pVHL binding to HIF-1α.[31] In addition, STAT3 could interact with p65 in the nucleus and recruit the p300 histone acetylase to the complex, leading to the prolongation of nuclear retention of p65. Furthermore, STAT3 and NF-κB could share the same binding sites in the regulatory regions of target genes.[32] Thus, the inhibition of HIF-1 and NF-κB by cucurbitacin B in the present work may be associated with the interference with the STAT3 pathway. Further studies are needed to clarify the mode of action of these compounds on HIF-1 and NF-κB signaling.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a UV-1601 spectrometer. NMR experiments were performed on a Varian-unity Inova-400 instrument. ESIMS were recorded on a Finnigan Navigator LC/MS/DS mass spectrometer and HRESIMS on a Mariner mass spectrometer. Preparative HPLC was performed using a Waters system with a 515 pump and a 2996 photodiode array detector.

Plant Material. The seeds of *T. kirilowii* were collected in Cheju, Korea, in January 2008 and identified by Mr. Youngtak Yang, Jeju Agriculture Institute, Jeju, Korea. A voucher specimen (No 080520) has been deposited in the herbarium of the Molecular Cancer Research Center, Korean Research Institute of Biosciences and Biotechnology, Daejeon, Korea.

Extraction and Isolation. The dry seeds of *T. kirilowii* (10 kg) were powdered and extracted with MeOH (3 × 10 L) at room temperature. The extract was concentrated and suspended in H_2O (3 L). The suspension was successively partitioned in *n*-hexane and $CHCl_3$ (each 3 × 2 L), and the organic layers were evaporated *in vacuo* to give brown residues of 92.0 and 29.5 g, respectively. The $CHCl_3$ part was chromatographed on a silica column eluted by a gradient of *n*-hexane−EtOAc (20:1 to 1:50 v/v) to afford five fractions, A1−A5

(each 600 mL). Fraction A3 was fractionated on a reversed-phase column (OSD-60-I40/63, YMC, Japan) with a mobile phase of MeOH−H_2O (2:1 v/v), giving five fractions, B1−B5 (each 200 mL). Fractions B2, B3, and B4 were subjected to preparative HPLC (YMC Pack Pro C18 column, 250 × 20 mm, 30% MeCN in H_2O, flow rate 10 mL/min) to give **4** (7.0 mg), **1** (5.0 mg), and **3** (10.5 mg), respectively. Compound **2** (20.0 mg) was obtained from fraction A4 by preparative HPLC (YMC Pack Pro C18 column, 250 × 20 mm, 35% MeCN in H_2O, flow rate 10 mL/min).

(*E*)-3-(4-Hydroxybenzylidene)-6-hydroxy-4-methoxybenzo-2(3*H*)-furanone (1): yellow solid; UV (MeOH) λ_{max} nm (log ε) 202 (3.83), 261 (3.45), 395 (3.75); ^{1}H NMR (400 MHz, methanol-d_4) δ 3.94 (3H, s, OMe), 6.18 (1H, d, J = 1.6 Hz, H-7), 6.27 (1H, d, J = 1.6 Hz, H-5), 6.82 (2H, d, J = 8.8 Hz, H-3′,5′), 7.91 (1H, s, H-10), 8.02 (2H, d, J = 8.8 Hz, H-2′,6′); ^{13}C NMR (100 MHz, methanol-d_4) δ 168.5 (s, C-2), 161.3 (s, C-6), 161.2 (s, C-4′), 158.0 (s, C-4), 155.5 (s, C-8), 141.3 (d, C-10), 135.2 (d, C-2′,6′), 127.8 (s, C-1′), 118.4 (s, C-3), 116.3 (d, C-3′,5′), 106.8 (s, C-9), 95.6 (d, C-5), 92.0 (d, C-7), 56.3 (q, OMe); ESIMS m/z 285.6 $[M + H]^+$; HRESIMS m/z 285.2914 (calcd for $C_{16}H_{13}O_5$, 285.2910).

Cell Culture. Human hepatocellular carcinoma Hep3B cells and human cervical cancer HeLa 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. Hypoxic cultures were 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.

HIF-1 Reporter Assay. The ability of compounds to inhibit HIF-1 activation in Hep3B cells was determined by a luciferase reporter assay as previously described.[33] 17-Desmethoxy-17-*N*,*N*-dimethylaminoethylaminogeldanamycin (17-DMAG, Calbiochem, La Jolla, CA) was used as a positive control.

NF-κB Reporter Assay. The NF-κB inhibitory activity of the isolated compounds on HeLa cells was evaluated by a secreted alkaline phosphatase (SEAP) reporter assay.[34] Celastrol (Calbiochem, La Jolla, CA) was used as a positive control.

ELISA Assay for VEGF Secretion. 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 compounds for 16 h under normoxic or hypoxic conditions. The VEGF levels in culture supernatant were determined with the DuoSet ELISA development kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacture's instruction.

Western Blot Analysis. To evaluate the level of HIF-1α proteins, total Hep3B cell lysates were analyzed as described[33] by western blotting using anti-HIF-1α monoclonal antibody. Protein loading was controlled by probing the membranes for topoisomerase-1 protein with an anti-topoisomerase-1 antibody.

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Supporting Information Available: HIF-1 activity (% inhibition) of the extracts and fractions from *T. kirilowi* and experimental details for HIF-1 and NF-κB reporter assays, ELISA assays, and western blot analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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