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Saururus cernuus Lignans - Potent Small Molecule Inhibitors of Hypoxia-Inducible Factor-1

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Abstract

Hypoxia-inducible factor-1 (HIF-1) represents an important tumor-selective therapeutic target for solid tumors. In search of novel small molecule HIF-1 inhibitors, 5400 natural product-rich extracts from plants, marine organisms, and microbes were examined for HIF-1 inhibitory activities using a cell-based reporter assay. Bioassay-guided fractionation and isolation, followed by structure elucidation, yielded three potent natural product-derived HIF-1 inhibitors and two structurally related inactive compounds. In a T47D cell-based reporter assay, manassantin B₁, manassantin A, and 4-*O*-methylsauroneol inhibited hypoxia-induced HIF-1 activation with IC₅₀ values of 3, 3, and 20 nM, respectively. All three compounds are relatively hypoxia-specific inhibitors of HIF-1 activation, in comparison to other stimuli. The hypoxic induction of HIF-1 target genes *CDKN1A*, *VEGF* and *GLUT-1* were also inhibited. These compounds inhibit HIF-1 by blocking hypoxia-induced nuclear HIF-1 α protein accumulation without affecting HIF-1 α mRNA levels. In addition, preliminary structure-activity studies suggest specific structural requirements for this class of HIF-1 inhibitors.

Keywords

Hypoxia; Breast Cancer; HIF-1 Inhibitor; Natural Product; Small Molecule; Dineolignan; Sesquiterpeneolignan; Manassantin; *Saururus cernuus*

Introduction

Gene regulation (selective activation and inactivation of genes) plays an important role in the etiology and progression of cancer. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor composed of the oxygen-regulated HIF-1 α and the constitutively expressed HIF-1 β subunits [1]. HIF-1 activates gene expression and promotes tumor cell survival under hypoxic

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conditions (reduced oxygen tension) [1]. In solid tumors, the degree of tumor hypoxia correlates with advanced disease stages and poor prognosis [2]. Clinical studies indicate that the overexpression of HIF-1 α is a negative indicator for tumor treatment outcome [3–5]. Results from preclinical studies support the concept of HIF-1 inhibition as an effective molecular target-based approach for suppression of tumor growth and enhancement of treatment outcomes for radiation and chemotherapy [6–10]. Currently, intense research efforts are directed at the discovery of small molecule HIF-1 inhibitors [1,11].

In order to discover novel small molecule HIF-1 inhibitors, natural product-rich plant, marine invertebrate, and microbe extracts were selected as the source of chemical diversity. The term "natural product" refers to low molecular weight organic compounds that are produced by the host organism, presumably fulfilling specialized biological roles. In contrast to the essential biochemicals produced by all living organisms, these "secondary metabolites" are often unique to a given species (or Genera) and are typically thought to serve a specific ecological role, such as chemical defense. Natural products have been a major source of new drugs for centuries and the range of biochemical diversity offered by natural products is far greater than that readily achieved through synthetic chemistry. Over 60% of currently approved anticancer agents are of natural origin (natural products or synthetic compounds based on natural product models) [12]. A T47D human breast tumor cell-based reporter assay was recently used to examine natural product-rich extracts for HIF-1 inhibitory activities [13]. Bioassay-guided chromatographic fractionation of an active extract from the aquatic plant *Saururus cernuus* yielded manassantin B and 4-*O*-demethylmanassantin B, two potent hypoxia-selective HIF-1 inhibitors that blocked HIF-1 activation at submicromolar concentrations [13]. *Saururus cernuus* L. (Saururaceae) is a perennial plant found throughout the eastern half of the United States and has been used as a medicinal plant by both Native Americans and early colonists [14]. The majority of the compounds isolated from *S. cernuus* are lignans, sesquiolignans, and dineolignans. In this present study, we describe the identification and characterization of one sesquiolignan and two dineolignan HIF-1 inhibitors from *S. cernuus*, and examine the structural requirements for *S. cernuus* lignans to inhibit hypoxia-induced HIF-1 activation in breast tumor cells.

Materials and Methods

General Experimental Procedures for Natural Product Chemistry

Acquisition of ¹H NMR, ¹H-¹H COSY, NOESY, HMBC, ¹³C NMR, and HMQC spectra; methods used to obtain HRESIMS and ESIMS data, optical rotation, and IR spectrum; equipment and supplies used to perform HPLC and TLC analysis were as previously described [13].

Plant Material Collection, Extraction, and Isolation

Collection, identification, and processing of *Saururus cernuus* samples were described [13]. Crude extract (16.6 g) was obtained by extracting dried powdered stems and leaves (172 g) with 50% CH₂Cl₂ in MeOH. A portion of the extract (11.0 g) was subjected to vacuum liquid chromatography (VLC, Si gel, 32–63 μ m, hexanes-EtOAc-MeOH, step gradient) to yield eight fractions. The second fraction (230 mg, eluted with CH₂Cl₂) was purified by NP-HPLC [Prodigy[®] Silica (3), 5 μ m, 250 \times 21.2 mm, 2% CH₂Cl₂ in hexanes, 15.0 mL min⁻¹, photodiode-array detection (PDA) monitored at 232 and 280 nm] to yield austrobailignan-5 (**5**, 80 mg). The fifth fraction (1.4 g, eluted with 50% hexanes in EtOAc) was separated using a C₁₈ solid phase extraction (SPE) cartridge (MeOH:H₂O step gradient) to obtain four subfractions. The first subfraction (440 mg) was subjected to repeated RP-HPLC (Prodigy[®] ODS-3, 5 μ m, 250 \times 21.2 mm, 30% CH₃CN:30% MeOH:40% H₂O, 9.0 mL min⁻¹, PDA detection monitored at 232 and 280 nm) and yielded verrucosin (**4**, 6 mg) and 4-*O*-

methylsaucerneol (**3**, 27 mg). Purification of the active constituents from the second subfraction (95 mg) was accomplished by RP-HPLC (Prodigy[®] ODS-3, 5 μ m, 250 \times 21.2 mm, 9.0 mL min⁻¹, PDA detection monitored at 232 and 280 nm), successively eluted first with 30% CH₃CN:30% MeOH:40% H₂O and then with 25% CH₃CN:25% MeOH:50% H₂O. This effort yielded the dineolignans manassantin B (**6**, 6.5 mg) and manassantin B₁ (**1**, 3.5 mg). The sixth fraction from the original VLC (862 mg, eluted with 100% EtOAc) was separated on Sephadex LH-20 with 100% MeOH to yield 14 subfractions. The fifth subfraction (150 mg) was first separated by RP-HPLC (Prodigy[®] ODS-3, 5 μ m, 250 \times 21.2 mm, 65% CH₃CN:35% H₂O, 9.0 mL min⁻¹, PDA detection monitored at 232 and 280 nm) and the major partially purified compound then separated by NP-HPLC [Prodigy[®] Silica (3), 5 μ m, 250 \times 21.2 mm, 5% MeOH in CH₂Cl₂, 13.0 mL min⁻¹, PDA detection monitored at 232 and 280 nm] to yield manassantin A (**2**, 35 mg).

Structure Data

Manassantin B₁ (**1**): white amorphous powder; $[\alpha]_D^{25}$ -51.7° (c 0.33, MeOH); UV (MeOH) λ_{\max} (log ϵ) 208 (7.76), 234 (4.27), 282 (3.80) nm; IR (film) ν_{\max} 3473, 2962, 2931, 1590, 1511, 1450, 1259, 1139, 1037, 935, 809, 736 cm⁻¹; ¹H NMR (C₆D₆, 400 MHz) δ 0.68 (6H, d, J = 5.5 Hz, H-9', 9"), 1.20 (3H, d, J = 6.2 Hz, H-9), 1.26 (3H, d, J = 6.4 Hz, H-9"), 2.15 (2H, m, H-8', 8"), 3.38 (3H, s, 3"-OCH₃), 3.41 (3H, s, 3-OCH₃), 3.42 (3H, s, 4-OCH₃), 3.44 (3H, s, 3'-OCH₃), 4.30 (1H, m, H-8"), 4.36 (1H, m, H-8), 4.85 (1H, d, J = 7.9 Hz, H-7), 4.94 (1H, d, J = 7.9 Hz, H-7"), 5.31 (2H, s, 3"-OCH₂O-4"), 5.48 (2H, d, J = 5.7 Hz, H-7', 7"), 6.61 (1H, d, J = 8.1 Hz, H-5), 6.67 (1H, d, J = 8.0 Hz, H-5"), 6.77 (1H, d, J = 8.0 Hz, H-6"), 6.89 (2H, d, J = 8.0, H-6', 6"), 6.92 (1H, d, J = 8.0, H-5"), 6.95 (2H, br, s, H-2', 2"), 6.96 (1H, d, J = 8.0, H-6), 7.04 (1H, d, J = 8.0, H-5'), 7.08 (1H, J = 2.0 Hz, H-2), 7.16 (1H, overlapped with solvent residual peak, H-2"); ¹³C NMR (C₆D₆, 100 MHz) δ (ppm): 14.1 (CH₃, C-9"), 15.5 (CH₃, C-9), 15.5 (CH₃, C-9"), 17.6 (CH₃, C-9), 45.0 (CH, C-8'), 45.0 (CH, C-8"), 56.0 (4C, CH₃, -OCH₃, C-3, 4, 4', 4"), 74.5 (CH, C-7"), 79.2 (CH, C-7), 83.0 (CH, C-8"), 83.8 (2C, CH, C-7', 7"), 84.9 (CH, C-8), 101.2 (CH₂, 3"-OCH₂O-4"), 107.9 (CH, C-2"), 108.5 (CH, C-5"), 111.3 (CH, C-6"), 111.4 (CH, C-6'), 112.1 (CH, C-5'), 112.5 (CH, C-5), 119.5 (CH, C-2"), 119.6 (CH, C-2'), 119.8 (CH, C-5"), 120.2 (CH, C-5'), 120.3 (CH, C-6"), 120.7 (CH, C-6), 134.2 (C, C-1), 135.4 (C, C-1"), 137.3 (C, C-1'), 137.4 (C, C-1"), 146.8 (C, C-4'), 147.6 (C, C-4"), 147.8 (C, C-3"), 148.6 (C, C-4"), 150.4 (C, C-3), 150.6 (C, C-4), 151.9 (C, C-3'), 152.4 (C, C-3"); HR-ESIMS [M+Na]⁺ m/z 739.3087 (calculated for [C₄₁H₄₈O₁₁ +Na], 739.3094).

Manassantin A (**2**): clear amorphous solid; $[\alpha]_D^{25}$ -98.4° (c 1.0, CHCl₃); HRESIMS m/z 755.3488 (calculated for C₄₂H₅₂O₁₁Na, 755.3407). Structure was assigned by 1D and 2D-NMR data, and the IR, UV, $[\alpha]_D$, ¹H and ¹³C NMR spectroscopic data are essentially identical to previously published values [15].

4-O-Methylsaucerneol (**3**): white amorphous solid; $[\alpha]_D^{25}$ -50.9° (c 0.7, CHCl₃); HR-ESIMS: (M+Na)⁺ m/z 575.2615 (calculated for C₃₂H₄₀O₈Na, 575.2621). ¹H and ¹³C NMR spectroscopic data are essentially identical to previously published values [15].

Verrucosin (**4**): white amorphous solid; $[\alpha]_D^{25}$ +20.6° (c 0.07, CHCl₃); HR-ESIMS: (M-H)⁻ m/z 343.1552 (calculated for C₂₀H₂₃O₅, 343.1545). ¹H and ¹³C NMR spectroscopic data are essentially identical to previously published values [16,17].

Austrobailignan-5 (**5**): light yellow oil; $[\alpha]_D^{25}$ -27.2° (c 2.5, CHCl₃); HR-ESIMS: (M+Na)⁺ m/z 349.1389 (calculated for C₂₀H₂₂O₄Na, 349.1416) [18].

Manassantin B (**6**): The isolation and spectroscopic identification of this compound was recently reported [13].

Cell Culture and Compound Treatment

Human breast carcinoma T47D cells (ATCC) were maintained in DMEM/F12 medium (JRH Biosciences) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone) and 0.5% penicillin/streptomycin (equivalent to 50 units/mL and 50 µg/mL, respectively, GIBCO). Test compounds were prepared as stock solutions in DMSO (Sigma) and stored at -80°C. The final concentration of DMSO is less than 0.5% (v/v) in all assays. The hypoxic conditions were achieved by purging a humidified modular incubator chamber (Billups-Rothenberg) with a hypoxic gas mixture (5% CO₂/1% O₂/94% N₂). In general, the cells were exposed to test compounds for 30 min under normoxic conditions prior to extended incubation.

T47D Cell-Based Reporter Assay

To monitor HIF-1 activity, a dual luciferase assay employing the pHRE3-TK-Luc reporter and an internal control pRL-TK (Promega) was performed as described [19].

RNA Extraction and Quantitative Real Time RT-PCR

Exponentially grown T47D cells were plated at the density of 0.9×10^6 cells/well of a 6-well plate (Corning) in 3 mL DMEM/F12 medium supplemented with 10% FBS and penicillin/streptomycin. Sixteen hours later, a serum-free DMEM/F12 medium supplemented with penicillin/streptomycin and each specific test compound was used to replace half of the conditioned media. Following incubation (30 min, 37°C), the exposure was prolonged for another 4 or 16 h under hypoxic conditions. Total RNA samples were extracted immediately following treatments with the RNeasy Mini Prep kit (QIAGEN). Synthesis of the first strand cDNAs, gene-specific primer sequences, quantitative real-time PCR reactions, and data analysis were described previously [19].

ELISA Assay for Human VEGF Protein

The plating of T47D cells, compound treatments, ELISA assay for secreted human VEGF proteins, and determination of the number of viable cells were the same as previously described [19].

Nuclear Extract Preparation and Western Blot Analysis

The experimental procedures were previously described in detail [13]. Final concentration of the solvent (DMSO) is 0.1% (v/v).

Mitochondrial Isolation and Respiration Studies

These studies were performed in the same manner as we have previously reported [20].

Statistical Analysis

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

Results and Discussion

Identification and Characterization of Novel HIF-1 Inhibitors from *Saururus cernuus*

A T47D cell-based reporter assay was used to monitor HIF-1 activity [13]. Bioassay-guided chromatographic fractionation of the *S. cernuus* lipid extract yielded three new HIF-1 inhibitors (**1**, **2**, **3**) and two structurally related inactive compounds (**4**, **5**) (Figure 1A). A combination of spectroscopic and spectrometric methods including Multi-Dimensional Nuclear Magnetic Resonance Spectroscopy (NMR) and High-Resolution Mass Spectrometry (HRMS) were used

to elucidate the chemical structures. The absolute configurations were defined by NMR analysis of Mosher ester derivatives. Compound **1** is a previously unreported stereoisomer of manassantin B that we now refer to as manassantin B₁. Compound **2** was found to be the *S. cernuus* dineolignan manassantin A, and compound **3** was determined to be the sesquiolignan 4-*O*-methylsaucerneol. Both inactive compounds **4** (verrucosin) and **5** (austrobailignan-5) are monolignans. The chemical structures of compounds **1–5** and manassantin B (**6**), which was used as a positive control, are shown in Figure 1A.

The effects of **1–5** on HIF-1 activity were examined and manassantin B (**6**) was used as a positive control. In a T47D cell-based dual luciferase reporter assay, no inhibition of hypoxia-induced HIF-1 activation (1% O₂, 16 h) was observed in the presence of either **4** (up to 1 μM) or **5** (up to 1 μM). Dose-response studies were performed to determine the effects of **1–3** on hypoxia-induced HIF-1 activation in T47D cells and the data are shown in Figure 1B. Compounds **1**, **2**, and the positive control **6**, all completely inhibited hypoxic activation of HIF-1 at the concentration of 10 nM. Compound **3** is about an order of magnitude less potent than **1**, **2**, and **6** at inhibiting hypoxia-induced HIF-1 activation. Since manassantins A (**2**) and B (**6**) inhibit hypoxic activation of HIF-1 with similar potency, it appears that the methylenedioxy moiety in manassantin B (**6**, IC₅₀ 2 nM) can be replaced by a pair of *O*-methyl groups, as in manassantin A (**2**, IC₅₀ 3 nM) with only a slight change in potency. Similarly, there is only a minor difference in potency between manassantin B (**6**, IC₅₀ 2 nM) and manassantin B₁ (**1**, IC₅₀ 3 nM). Therefore, the difference in relative configuration of at least one of the two side chains in manassantin B (*threo*) and B₁ (*erythro*) has only a minor influence on the potency of HIF-1 inhibition. A more dramatic effect was observed with the absence of one side chain phenylpropyl unit, as in the sesquiolignan 4-*O*-methylsaucerneol (**3**). This reduced the potency of **3** by about 90% (IC₅₀ 20 nM), relative to that of manassantin B (**6**, IC₅₀ 2 nM). The most striking difference was observed with the two monolignans verrucosin (**4**) and austrobailignan-5 (**5**) that show virtually no HIF-1 inhibition, even at 1 μM, a concentration 500-times that of the IC₅₀ for manassantin B (**6**). This suggests that the absence of the second hydroxylated side chain segment not only reduces the HIF-1 inhibitory activity of *S. cernuus* lignans, but for all practical purposes, renders these compounds essentially inactive.

Two conditions that are commonly used to activate HIF-1 are those of physiological hypoxia [21] and chemical hypoxia [22,13]. To discern the specificity of HIF-1 inhibition, experiments were performed to determine the effects of **1–3** on iron chelator-induced HIF-1 activation in T47D cells (10 μM 1,10-phenanthroline, 16 h). As shown in Figure 1C, none of the compounds exhibited greater than 50% inhibition of 1,10-phenanthroline-induced HIF-1 activation at the concentrations tested. This observation suggests that, like the positive control **6**, **1–3** may function as hypoxia-selective HIF-1 inhibitors. It is most likely that these compounds target a process or set of processes specific to the activation of HIF-1 by hypoxia.

Effects of *S. cernuus* Lignans on HIF-1 Target Gene Expression

More than seventy genes have been identified to be cellular targets of HIF-1 [1]. Most of these genes are regulated by HIF-1 in a cell type-specific manner, while a few genes are induced in most cell types. The effects of manassantins on hypoxic induction of HIF-1 target genes (*CDKN1A*, *GLUT-1*, and *VEGF*) were examined in T47D cells and these results are shown in Figure 2. All three genes are induced in most cell types upon HIF-1 activation. In T47D cells, hypoxic exposure (1% O₂, 16h) increased the expression of *CDKN1A*, *GLUT-1*, and *VEGF* at the mRNA level by 1.4-, 5-, and 6-fold, respectively. Compounds **1**, **2**, and **6** (at 30 nM each) blocked the hypoxia-induced increase in *CDKN1A* mRNA, while **4** did not affect the induction (Figure 2A). These compounds exerted similar effects on hypoxia-induced increases in *GLUT-1* (Figure 2B) and *VEGF* (Figure 2C) mRNA levels. Compounds **1**, **2**, and **6** inhibited

the hypoxia-induced increase in GLUT-1 and VEGF mRNA levels, while compound **4** was inactive. Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors known [23]. Inhibition of VEGF gene expression/function has been pursued as a noncytotoxic approach for the treatment of cancer [23]. Since secreted VEGF proteins are the bioactive forms and hypoxia is one of the major physiological stimuli for VEGF production, the effects of **1–3** on hypoxic induction of secreted VEGF proteins were also examined in T47D cells (Figure 3). Compounds **1**, **2** and the positive control **6**, blocked the hypoxia-induced increase in secreted VEGF proteins at the lowest concentration tested (10 nM). In the presence of **3**, partial inhibition (60%) was observed at the lower concentration (10 nM), while complete inhibition was achieved at the higher concentration (100 nM). When tested at 100 nM, none of the compounds interfered with the ELISA assay using recombinant human VEGF₁₆₅ protein as the standard (no greater than 15% difference, data not shown).

Manassantins and 4-O-Methylsauroneol Inhibit HIF-1 by Blocking Nuclear HIF-1 α Protein Accumulation

In general, the oxygen-regulated HIF-1 α subunit determines HIF-1 activity [21]. Under normoxic conditions, HIF-1 α protein is post-translationally modified and rapidly degraded by the ubiquitin-proteasome pathway [24–26]. Under hypoxic conditions, stabilization and accumulation of nuclear HIF-1 α protein precedes the activation of HIF-1 [24–26]. In T47D cells, hypoxic exposure (1% O₂, 4 h) leads to the accumulation of nuclear HIF-1 α protein without affecting the constitutively expressed HIF-1 β protein (Figure 4A). Compounds **1–3** (and the positive control **6**) blocked the hypoxia-induced accumulation of nuclear HIF-1 α protein (Figure 4A). The structurally related, yet inactive, compound **4** did not affect the induction of nuclear HIF-1 α protein by hypoxia (Figure 4A). No significant changes in nuclear HIF-1 β protein levels were observed following treatments (Figure 4A). The effects of test compounds on HIF-1 α expression at the mRNA level were also examined. As shown in Figure 4B, none of the compounds (**1–4**, and **6** at 30 nM each) affected HIF-1 α mRNA levels under hypoxic conditions (1% O₂, 4 h). Thus, compounds **1–3** most likely inhibit HIF-1 by regulating the availability of HIF-1 α protein. There are at least three possible scenarios that can contribute to this outcome: 1) inhibition of HIF-1 α protein synthesis; 2) degradation of HIF-1 α protein; and 3) blockade of HIF-1 α protein nuclear translocation. However, the following two observations suggest that it is unlikely that these HIF-1 inhibitors function by blocking HIF-1 α protein synthesis or nuclear translocation: 1) these compounds did not inhibit 1,10-phenanthroline-induced HIF-1 activation with the same potency (Figure 1C); and 2) manassantin B (**6**) did not inhibit 1,10-phenanthroline-induced accumulation of nuclear HIF-1 α protein [13].

To test the hypothesis that HIF-1 inhibitors such as manassantin B (**6**) can promote HIF-1 α protein degradation, the effect of **6** on nuclear HIF-1 α protein accumulation was examined in the presence of MG132. The compound MG-132 is a membrane permeable and reversible inhibitor of the proteasome and has been shown to block proteasome-mediated degradation of HIF-1 α protein and induce nuclear HIF-1 α protein accumulation [27,28]. Under normoxic conditions, MG-132 (10 μ M, 4 h) induced the accumulation of nuclear HIF-1 α protein in T47D cells, and this induction was blocked by manassantin B (**6**) (Figure 4C). Under hypoxic conditions, MG132 enhanced the induction of HIF-1 α protein by hypoxia, and manassantin B (**6**) inhibited the induction, even though the inhibition was not complete (Figure 4C). One obvious explanation is that manassantin B can effectively overcome the reversible proteasome inhibition imposed by MG132 under normoxic conditions but not under hypoxic conditions. However, additional studies will be required to confirm this hypothesis.

Effects of *S. cernuus* Lignans on Mitochondrial Respiration

Among the known HIF-1 inhibitors that we have examined, rotenone exhibited similar effects as those observed for the active *S. cernuus* neolignans on the induction of nuclear HIF-1 α protein by various stimuli. In T47D cells, rotenone (1 μ M) inhibited the accumulation of nuclear HIF-1 α protein induced by hypoxia (1% O₂, 4 h), MG-132 (10 μ M, 4 h), MG-132 plus hypoxia, but not by 1,10-phenanthroline (10 μ M, 4 h) (data not shown). Rotenone is a potent inhibitor of mitochondrial electron transport and has also been shown to inhibit the activation of HIF-1 [29]. Recent studies have shown that mitochondrial inhibitors block hypoxic activation of HIF-1 by increasing intracellular oxygen levels [30]. To test the hypothesis that these active *S. cernuus* neolignans block HIF-1 activation via mitochondrial inhibition, the effects of **1–4** and **6** on mitochondrial respiration were examined, and these results are shown in Figure 5. Compounds **1**, **2**, **3**, and **6** inhibited mitochondrial oxygen consumption with IC₅₀ values of 320 nM, 600 nM, 260 nM, and 250 nM, respectively. No effects on oxygen consumption by mitochondria were observed in the presence of **4** (highest concentration tested: 9 μ M). Addition of succinate did not relieve the inhibition exerted by **1**, **2**, **3**, and **6** on mitochondria, suggesting that these compounds act as inhibitors of mitochondrial electron transport chain complex I. At present time, it can not be conclusively determined whether the precise mechanism of action for these potent HIF-1 inhibitors is mediated through the inhibition of mitochondrial oxygen consumption. Several other natural product-based mitochondria inhibitors suppress both HIF-1 activation and mitochondrial function with similar potency [20,30].

Previously reported activities of manassantins A and B include a tranquilizer-like activity in mice (manassantin A) [31,32], inhibition of NF κ B activation (manassantins A and B) [33, 34], inhibition of human acyl-CoA: cholesterol acyltransferase-1 and -2 (manassantins A and B) [35], inhibition of PMA-induced cell aggregation (manassantins A and B) [36], and inhibition of HIF-1 activation (manassantin B) [13]. Most of these activities that have been demonstrated in either cell-based or enzyme-based assays require manassantin concentrations that are in the micromolar range. Manassantins A and B were shown to inhibit PMA-induced aggregation of HL-60 cells with MICs of 1.0 and 5.5 nM, while concentrations at least ten-fold higher are required for each manassantin to block PMA-induced ICAM expression (IC₅₀s: >10 nM, and MICs: 10–100 nM)[36]. However, it was suggested that manassantins A and B inhibit cell aggregation by blocking ICAM-1 expression [36]. In our previous report, isolated samples of both manassantin B (**6**) and 4-*O*-demethylmanassantin B were shown to block hypoxia-induced HIF-1 activation in T47D cells with IC₅₀s of 3 and 30 nM, respectively [13]. Inhibitory effects of manassantin B on hypoxia-induced HIF-1 α protein accumulation were also observed in other cell lines (Hep3B and MDA-MB-231) with similar potency (data not shown). Therefore, it is unlikely that the potent HIF-1 inhibitory activity of manassantin B is limited to T47D cells.

As mentioned, intense research efforts are currently underway in many laboratories to discover and develop new strategies to inhibit HIF-1. Among the small molecule HIF-1 inhibitors identified, many of these compounds (i.e. 2-methoxyestradiol [37], taxol [37], geldanamycin [27], rapamycin [38], topotecan and camptothecin analogues [39], and PX-478 [40]) are also known to exert their anticancer effects through other mechanisms. Most of these compounds were discovered initially due to their effects on other cellular targets, and their inhibitory effects on HIF-1 were later identified. In the published literature, the sources of chemicals used in screening efforts to discover new HIF-1 inhibitors can be classified into two general categories: 1) libraries of pure compounds [39,41,42]; and 2) libraries of natural product-rich extracts [13]. Among the HIF-1 inhibitors discovered from such screening efforts, the most potent inhibitors of HIF-1 activation (with IC₅₀ values in the nM range) are all natural products or compounds based on natural product scaffolds (e.g. manassantins A, B [13], B₁, 4-*O*-

demethylmanassantin B [13], 4-*O*-methylsauroneol, chetomin [41], topotecan [39], and camptothecin analogues [39]). The recently identified natural product-based synthetic analog 103D5R, inhibits HIF-1 activation at high concentrations (IC₅₀ of 35 μM), but is also cytostatic [42]. The unique properties of manassantin B and related compounds include their relative selectivity towards hypoxia-induced HIF-1 activation (in contrast to iron chelator-induced HIF-1 activation), and that they exert HIF-1 inhibitory effects at concentrations far below cytotoxic concentrations (supporting information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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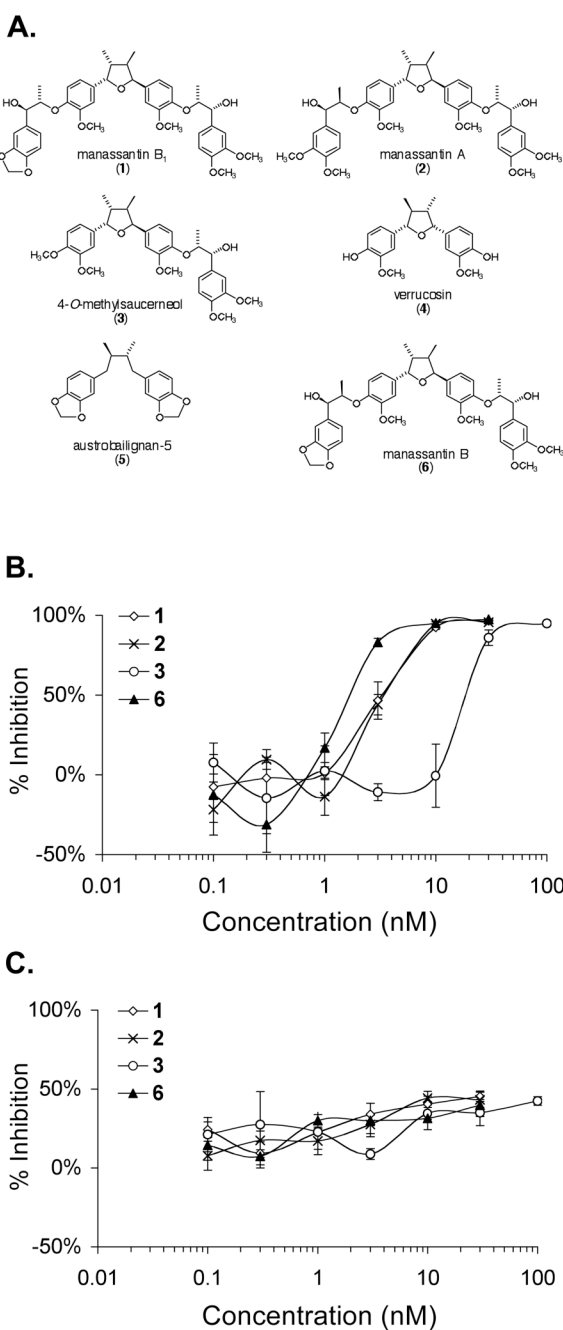


Figure 1.

Chemical structures of six *Saururus cernuus* lignans and their effects on HIF-1 activity in T47D cells. The structures, trivial names, and numbers are shown in (A). Dose response studies were performed in T47D cells using a dual luciferase reporter assay to determine the effects of **1–3** on HIF-1 activation by hypoxia (B) and by 10 μ M 1,10-phenanthroline (C). Compound **6** was included as a positive control. The cells were exposed to test compounds for 30 min prior to hypoxic exposure (16 h, 1% O₂) or 1,10-phenanthroline treatment under normoxic conditions (16 h, 95% Air). Data are presented as averages from one representative experiment performed in triplicate and the error bars represent standard error.

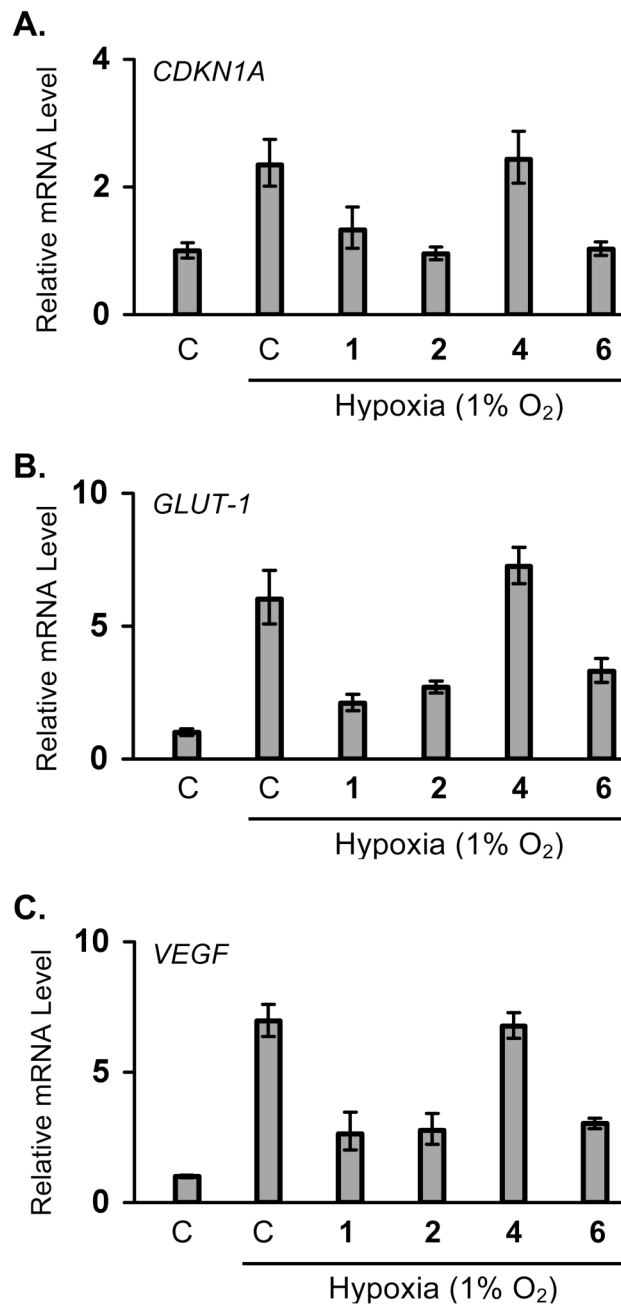


Figure 2. Manassantins inhibit hypoxic induction of HIF-1 target genes *CDKN1A*, *GLUT-1*, and *VEGF* in T47D cells. Quantitative real time RT-PCR analysis was performed to determine *CDKN1A* (A), *GLUT-1* (B), and *VEGF* (C) mRNA levels following treatments with **1**, **2**, **4**, and **6** (30 nM each) under hypoxic conditions (1% O₂) for 16 h. "C" represents media only. Data shown are relative expression levels (mean ± SD) of target genes normalized to that of an internal 18S rRNA control, determined from one representative experiment performed in triplicate.

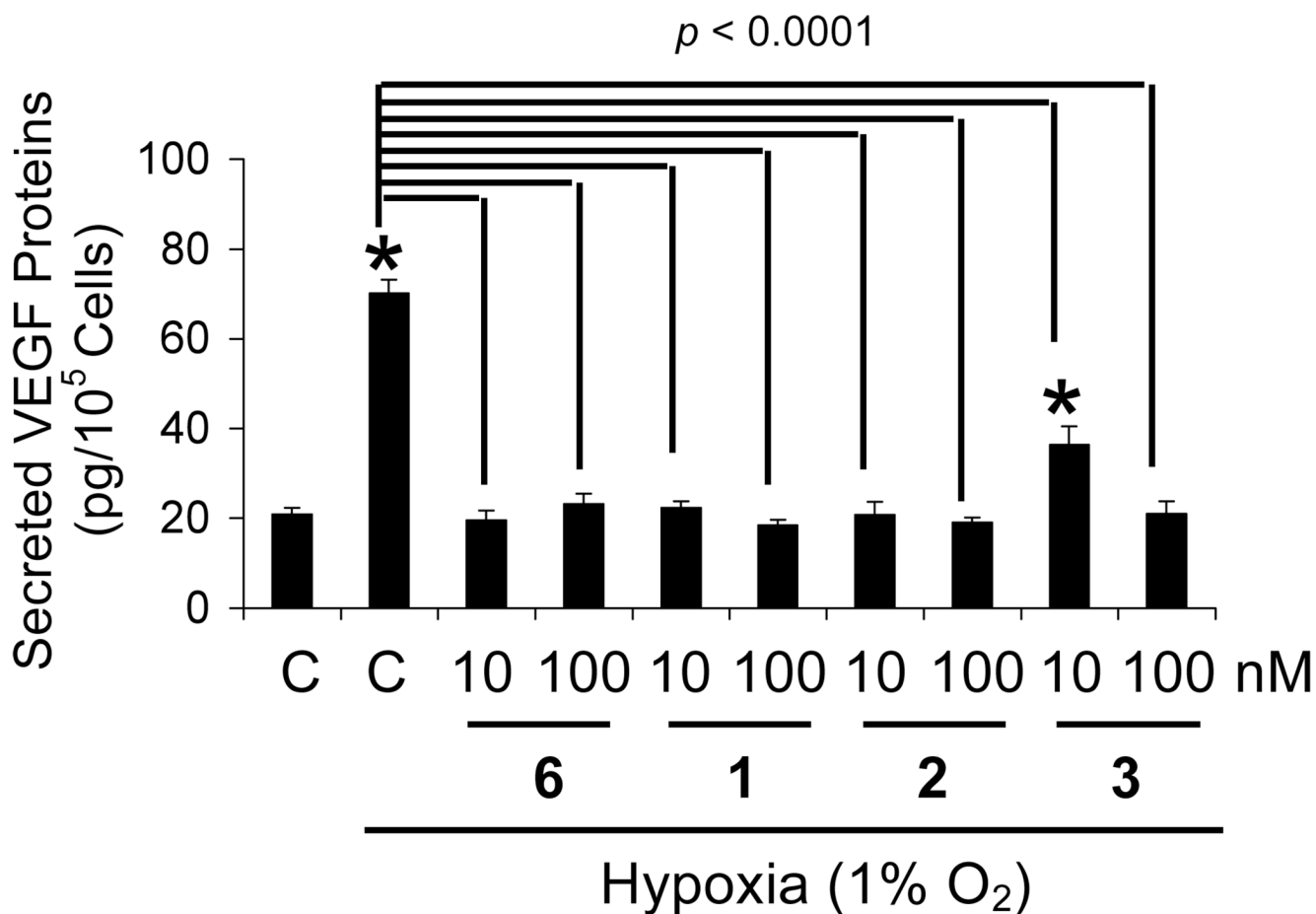


Figure 3.

Manassantins and 4-*O*-methylsaucerneol block the induction of secreted VEGF proteins (VEGF₁₂₁ and VEGF₁₆₅) by hypoxia in T47D cells. Following compound treatment under hypoxic conditions (1% O₂) for 16 h, levels of secreted VEGF proteins in the conditioned media were determined by ELISA and normalized to the number of viable cells. The control "C" represents media only. Data shown are mean \pm SE from one representative experiment performed in triplicate. An asterisk (*) indicates $p < 0.05$ when compared to the normoxic control. The p values between the hypoxic control and the compound treatments are indicated.

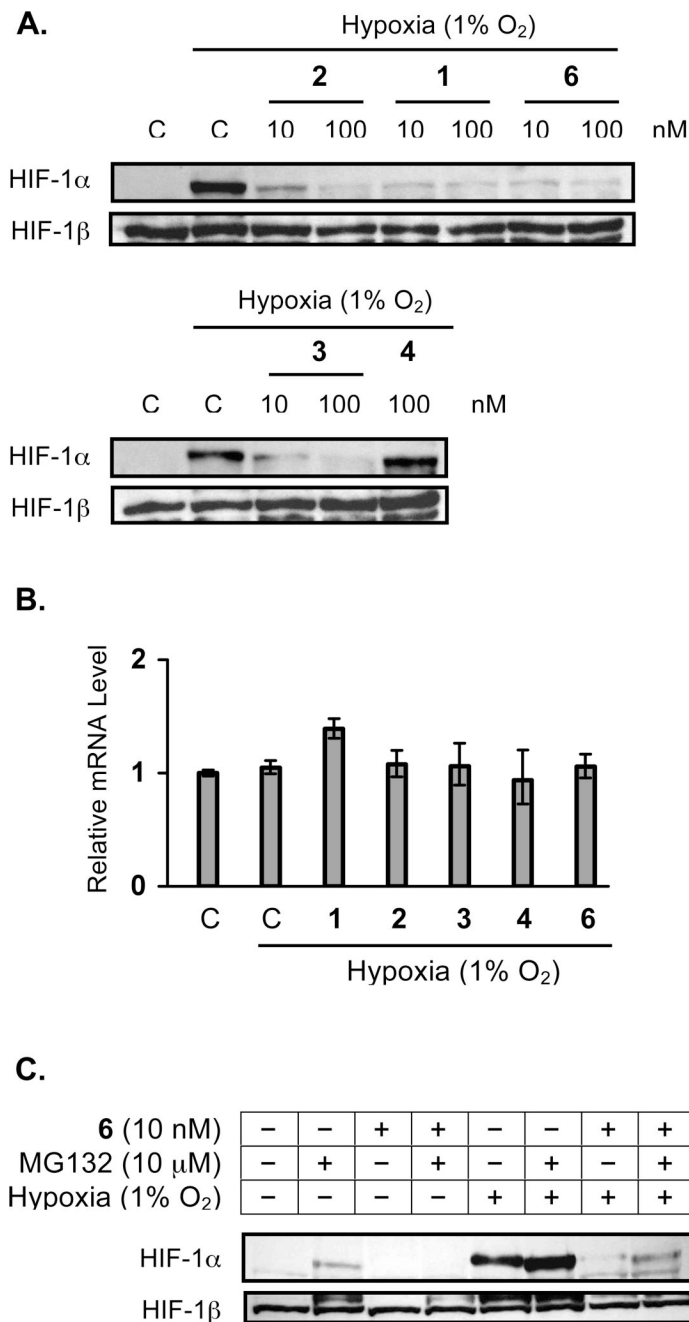


Figure 4. Manassantins and 4-*O*-methylsauerceol inhibit HIF-1 activation by preventing the accumulation of nuclear HIF-1α protein. After hypoxic exposure (1% O₂, 4 h) in the presence of test compounds, levels of HIF-1α and HIF-1β proteins in the nuclear extract samples from T47D cells were determined by Western blot (A). The control "C" represents media only. Quantitative real time RT-PCR analysis was employed to determine the effects of **1–4** and **6** (30 nM each) on HIF-1α mRNA levels under hypoxic conditions (1% O₂, 4 h). Data shown are relative expression levels of HIF-1α mRNA, determined from one representative experiment performed in triplicate (B). Effects of **6** on the stabilization of nuclear HIF-1α protein induced by a proteasome inhibitor (MG-132) under normoxic (95% Air, 4 h) and

hypoxic conditions (1% O₂, 4 h) were examined by Western blot (C). Levels of nuclear HIF-1 β protein detected by Western blot were also shown (C).

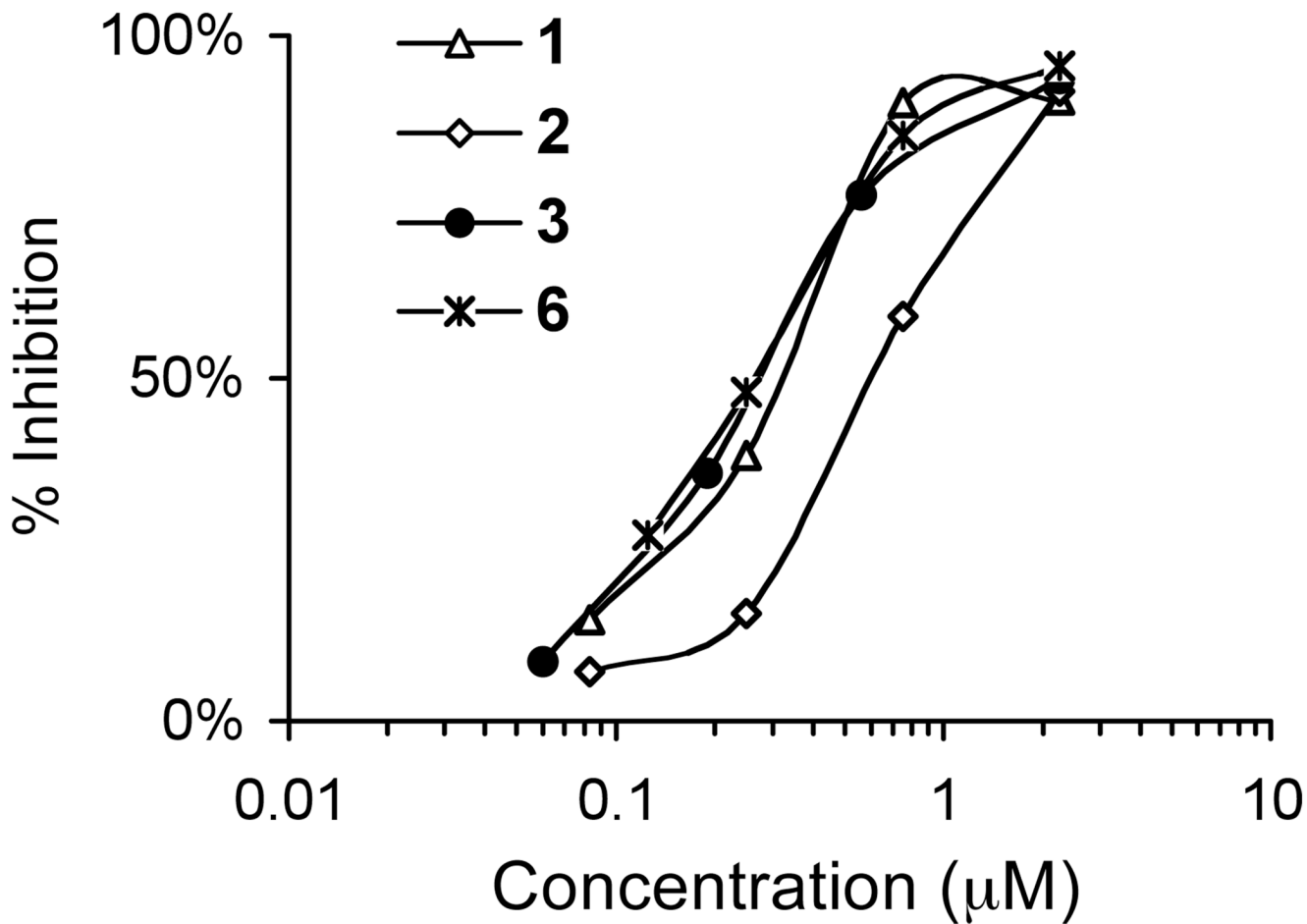


Figure 5.

Effects of compounds 1–3 and 6 on the consumption of oxygen by mitochondria. The mitochondrial oxygen consumption rate (nmole O₂/min/mg protein) was measured in the presence and absence of test compounds *in vitro*. Data shown are averages from two independent assays, presented as percentage inhibition in comparison to the untreated control.