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Analysis of HIF-1 inhibition by manassantin A and analogues with modified tetrahydrofuran configurations

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Abstract

We have shown that manassantin A downregulated the HIF-1 α expression and inhibited the secretion of VEGF. We have also demonstrated that the 2,3-*cis*-3,4-*trans*-4,5-*cis*-configuration of the tetrahydrofuran is critical to the HIF-1 inhibition of manassantin A.

Molecular oxygen (O₂) is required for aerobic metabolism to maintain intracellular bioenergetics and to serve as an electron acceptor in many organic and inorganic reactions.¹ Hypoxia, usually defined as $\leq 2\%$ of O₂, occurs in a variety of pathological conditions, including stroke, tissue ischemia, inflammation, and tumor growth.² Mammalian tissues have developed a number of essential mechanisms to cope with the stress of hypoxia. Among these coping mechanisms is the response mediated by the hypoxia-inducible transcription factor 1 (HIF-1). It is a basic helix-loop-helix (bHLH)-PER-ARNT-SIM (PAS) family protein that forms a heterodimer with its α and β subunits and acts as a transcription factor.³ There are two additional HIF-1 α -related bHLH-PAS proteins: HIF-2 α and HIF-3 α .⁴ Like HIF-1 α , they also bind to HIF-1 β (ARNT) for activation. HIF-1 is a main regulator of hypoxia since it activates more than 60 genes involved in angiogenesis (VEGF), glucose transport (GLUT1), glycolytic pathways (LDHA), ROS signals (iNOS), and erythropoiesis (EPO), as well as a number of other processes.⁵

Through HIF-1, tumors adapt to hypoxia by increasing angiogenesis and metastatic potential, altering apoptosis, and regulating metabolism.⁶ These adaptations make tumors more aggressive and treatment-resistant resulting in poor patient prognosis.⁷ Immunohistochemical analyses have revealed that HIF-1 is overexpressed in many human cancers.⁸ HIF-1 overexpression is not only involved in tumor progression but also associated with resistance to radiation⁹ and chemotherapy.¹⁰ It has been shown that inhibition of HIF-1 activity suppresses tumor growth, destroys blood vessels, enhances tumor apoptosis, and increases radiosensitivity,¹¹ making HIF-1 a good potential target for anti-cancer treatment.

Due to the importance of HIF-1 in tumor development and progression, a considerable amount of effort has been made to identify HIF-1 inhibitors for treatment of cancer.¹² A variety of anticancer drugs, most of which were not developed as HIF-1 inhibitors, have been reported

to inhibit HIF-1. However, these compounds possess relatively low HIF-1 inhibitory activity ($\geq$ micromolar range). In addition, most of them lack the desired selectivity for the HIF-1 signaling pathway or toxicity profiles required for a useful therapeutic agent. The dineolignans manassantin A (**1**), manassantin B (**2**), manassantin B₁ (**3**), and 4-*O*-demethylmanassantin B (**4**) (Figure 1), isolated from the aquatic plant *Saururus cernuus* L. (Saururaceae), have been shown to be potent inhibitors of HIF-1.¹³ However, their molecular mechanisms of action have yet to be established. Since manassantins may sensitize cancer cells to chemo-and/or radiotherapy by HIF-1 inhibition,^{11c,14} use of manassantins in combination with other cytotoxic drugs and/or radiation has great potential for therapeutic applications.

In broad connection with our interest in the stereoselective synthesis of substituted tetrahydrofurans,^{15,16} we recently completed the synthesis of **1** and **2** via a direct nucleophilic addition of an organozinc reagent to a 2-benzenesulfonyl cyclic ether to synthesize the 2,3-*cis*-3,4-*trans*-4,5-*cis*-tetrahydrofuran moiety of **1** and **2**.¹⁷ In addition, we also showed that the (*R*)-configuration at C-7 and C-7''' is not critical for HIF-1 inhibition and that the hydroxyl group at C-7 and C-7''' can be replaced with carbonyl group without significant loss of activity.

Herein, we present initial biological data demonstrating the significant potential of **1** as a potent HIF-1 inhibitor with little cytotoxicity. In addition, we describe the synthesis and conformation–activity relationship study of tetrahydrofuran core analogues of **1** to characterize the effect of tetrahydrofuran conformation on HIF-1 inhibitory activity.

Previously, we reported that **1** exhibited a highly potent level of HIF-1 inhibitory activity in a luciferase-reporter based assay (IC_{50} = 1–10 nM).¹⁷ Based on the luciferase assay data, we used Western blots to further confirm the HIF-1 inhibitory activity of **1**. 4T1 cells, a mouse mammary carcinoma, were grown under hypoxic conditions (0.5% O₂) for 24 h with various concentrations (0, 1, 10, 100 nM, and 1 μ M) of **1**. Western blots were performed on nuclear extracts as reported.^{11b} A dose-response study revealed that exposure of 4T1 cells to **1** at concentrations higher than 10 nM for 24 h significantly inhibited hypoxia-induced expression of the HIF-1 α protein (Figure 2-a).

To determine if **1** also inhibits chemically-induced HIF-1 α expression,¹⁸ we treated 4T1 cells with 240 μ M of CoCl₂ for 24 h and carried out Western blots with nuclear extracts. HIF-1 α expression induced by CoCl₂ was inhibited by **1** (100 nM) (Figure 2-b) indicating that **1** inhibits chemically induced HIF-1 α expression as well as hypoxia-induced HIF-1 α expression. It should be noted that **1** was reported to have no significant effect on iron chelator-induced HIF-1 activation in T47D cells (10 μ M 1,10-phenanthroline, 16 h).^{13c} To examine if the HIF-1 inhibition by **1** is cell-type specific, we carried out the same experiment with MDA-MB-231, a human breast cancer cell line, and observed the same inhibition effect, which showed that the HIF-1 inhibition effect by **1** is not cell-type specific (Figure 2-b).

As stated earlier, more than 60 genes have been identified as targets of HIF-1. Vascular endothelial growth factor (VEGF) is a gene that is highly involved in tumor progression as a pro-angiogenic factor. The effects of **1** on HIF-1 regulated VEGF secretion were examined in 4T1 cells using ELISA. Cells were incubated under hypoxia (0.5% O₂ for 24 h) with various concentrations (0, 1, 10, 100 nM, and 1 μ M) of **1**. Cell culture supernates were collected, and VEGF levels in supernates were measured by a commercially available kit (R&D systems, Minneapolis, MN). As we observed from HIF-1 expression, VEGF induced by hypoxia was significantly inhibited by **1** at concentrations higher than 10 nM (Figure 3).

Cytotoxicity of **1** was examined using the MTS assay, a standard colorimetric cytotoxicity assay (see Supporting Information for details). 4T1 cells were seeded in a 96-well plate and incubated with serially diluted **1** (0–10 μ M) for 24 h. Up to the highest concentration examined

(10 μ M), cells had ~70% survival rate. Considering that **1** completely inhibited the expression of HIF-1 at the concentration ≤ 100 nM, **1** possesses a significant therapeutic window (IC_{50} (cytotoxicity)/ IC_{50} (HIF-1 inhibition) ≥ 100).

The configuration of **1** is largely determined by the 2,3-*cis*-3,4-*trans*-4,5-*cis*-configuration of the tetrahydrofuran core. We hypothesized that the overall conformation should be an important determinant for the binding mode and affinity toward molecular target(s), potency, and HIF-1 signaling specificity of **1**. To test the hypothesis, we prepared and evaluated manassantin A analogues with modifications in tetrahydrofuran configuration. These analogues were easily prepared through the procedures previously reported by our group (Scheme 1).^{15,17}

The synthesis of manassantin A analogues with 2,3-*cis*-3,4-*trans*-4,5-*trans*- and 2,3-*trans*-3,4-*trans*-4,5-*trans*-tetrahydrofuran cores (**11** and **13**) was accomplished as described in Scheme 1. Briefly, the 2,3-*cis*-3,4-*trans*-4,5-*trans*-tetrahydrofuran **7a** was prepared via $BF_3 \cdot OEt_2$ -promoted deoxygenation of cyclic hemiketal **6**¹⁵ followed by stereoselective reduction of the oxocarbenium ion intermediate. Deprotection of the Bn and TBS groups, BEMP-mediated coupling, and polymer-supported BH_4 -reduction completed the synthesis of **11**.¹⁹ Compound **13** was prepared via $BF_3 \cdot OEt_2$ -promoted epimerization/reductive deoxygenation followed by deprotection, BEMP-mediated coupling, and polymer-supported BH_4 -reduction.²⁰

To determine HIF-1-inhibitory activity of **11** and **13**, we used a luciferase-reporter based assay as a primary screen. For this assay, we used 4T1-ODD-Luc cells²¹ stably transfected with the oxygen-dependent-degradation (ODD) domain of HIF-1 α and a firefly luciferase reporter. This ODD-Luc reporter contains a CMV promoter, which is constitutively active. Since its ODD domain is identical to that of HIF-1, it enables us to directly detect the stability of HIF-1. Cells were seeded in the 24-well plate at a density of 10^5 cells/well. After 16-hour incubation, cells were treated with 240 μ M of $CoCl_2$ and serially diluted compounds for 24 h. Since luciferase requires O_2 for its activity but the ODD-Luc is highly sensitive to reoxygenation, we induced the HIF-1 expression by $CoCl_2$, not by hypoxia to accurately determine the effect of the compounds on HIF-1 stability. Luciferase signals were detected and quantified as relative light units (RLUs). The ODD-Luc assay to assess HIF-1 inhibitory activity of **11** and **13** revealed that **11** was nearly inactive and **13** was less active than **1** by 10-fold ($IC_{50} = 47$ nM) (Figure 4). Based on these results, the 2,3-*cis*-3,4-*trans*-4,5-*cis*-configuration of the tetrahydrofuran core is critical for HIF-1 inhibition.

To further characterize the effect of tetrahydrofuran conformation of **1**, **11**, and **13** on the HIF-1 inhibition, we optimized the conformations of truncated structures²² using density functional theory (B3LYP)²³ at the 6-31G* level (Gaussian 03, D.02 version²⁴). As shown in Figure 5, compound **14** adopted a nearly linear conformation, but compound **15** adopted a bent-shaped conformation remarkably different from that of **14**. However, in one of the two possible orientations for **16** (Overlay II), the conformation was relatively close to that of **14** (Figure 5-c and d)²⁵ indicating that the linear-shaped conformation resulting from the 2,5-*trans*-configuration is critical to the HIF-1 inhibition. Thus, designing a ligand that mimics the overall conformation of **1** may improve the potency and selectivity toward the hypoxia signaling pathway.

In summary, we have shown that **1** downregulated the HIF-1 α expression and inhibited the secretion of VEGF. We have also demonstrated that the 2,3-*cis*-3,4-*trans*-4,5-*cis*-configuration of the tetrahydrofuran is critical to the HIF-1 inhibition of **1**. This conformation-activity relationship study may help to identify structural motifs required for HIF-1 inhibition and allow structural modifications to increase specificity and decrease off-target effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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19. For 11: ^1H NMR (400 MHz, CDCl_3) δ 6.80–7.09 (m, 12 H), 5.13 (d, $J = 8.4$ Hz, 1 H), 4.63 (d, $J = 8.0$ Hz, 1 H), 4.62 (d, $J = 8.0$ Hz, 1 H), 4.42 (d, $J = 9.2$ Hz, 1 H), 4.06–4.14 (m, 2 H), 4.09 (br s, 2 H), 3.85–3.92 (m, 18 H), 2.23–2.30 (m, 1 H), 1.76–1.84 (m, 1 H), 1.16 (d, $J = 6.0$ Hz, 3 H), 1.15 (d, $J = 6.0$ Hz, 3 H), 1.08 (d, $J = 6.4$ Hz, 3 H), 0.67 (d, $J = 7.2$ Hz, 3 H); LRMS (FAB) found 732.4 [calcd for $\text{C}_{42}\text{H}_{52}\text{O}_{11}$ (M) $^+$ 732.4].
20. For 13: ^1H NMR (400 MHz, CDCl_3) δ 6.96–7.01 (m, 4 H), 6.91 (s, 2 H), 6.90 (dd, $J = 8.4, 1.2$ Hz, 4 H), 6.81 (d, $J = 8.0$ Hz, 2 H), 4.65 (d, $J = 9.2$ Hz, 2 H), 4.62 (d, $J = 8.4$ Hz, 2 H), 4.05–4.13 (m, 4 H), 3.92 (s, 6 H), 3.86 (s, 6 H), 3.85 (s, 6 H), 1.78–1.81 (m, 2 H), 1.14 (d, $J = 6.4$ Hz, 6 H), 1.06 (d, $J = 5.6$ Hz, 6 H); HRMS (FAB) found 732.3492 [calcd for $\text{C}_{42}\text{H}_{52}\text{O}_{11}$ (M) $^+$ 732.3510].
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22. To avoid any unnecessary complications and/or exaggeration by the flexible side chains, truncated structures of 1, 11, and 13 were used instead of the full structures. Initial geometries were determined by conformational search based on molecular mechanics (MMFF).
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25. Optimization of the full structures of 1, 11, and 13 using density functional theory (B3LYP) at the 6–31G* level provided the same conclusion (see Supporting Information for details).

Figure 1.
Structure of dineolignans from *Saururus cernuus*.

Figure 2.

Inhibition of HIF-1 α expression by **1**. (a) After treating 4T1 cells under hypoxia (0.5% O₂, 24 h) with and without **1**, HIF-1 α expression was evaluated using Western blots. By comparison with loading control (histone H1), nuclear expression of HIF-1 α was significantly inhibited by **1** at concentrations higher than 10 nM. (b) In 4T1 and MDA-MB-231 cells, HIF-1 α expression induced by CoCl₂ (240 μ M, 24 h) was also inhibited by **1**.

Figure 3.

Inhibition of VEGF secretion by **1**. Inhibition of VEGF was determined in 4T1 cells after hypoxia treatment (0.5% O₂, 24 h). After treatment with and without **1**, cell culture supernates were collected, and VEGF secretion was measured using ELISA. Compound **1** significantly inhibited VEGF at concentrations higher than 10 nM (p<0.001).

Figure 4.
Inhibition of HIF-1 by **11** and **13**.

Figure 5.

Optimized conformations of truncated structures of **1**, **11**, and **13** (a) Truncated structures (**14–16**) (b) Overlay I (c) Overlay II: front view (d) Overlay II: side view (**14** in black, **15** in red, **16** in blue, and tetrahydrofurans in green).

Scheme 1.

(a) $\text{Ar}^2 \text{Li}$, THF, -78°C , 40 min, 70%; (b) $\text{BF}_3 \cdot \text{OEt}_2$, NaBH_3CN , CH_2Cl_2 , -78°C , 30 min, 99%; (c) $\text{BF}_3 \cdot \text{OEt}_2$, CH_2Cl_2 , -78 to -20°C , 2 h; then, NaBH_3CN , -78°C , 30 min, 96%; (d) H_2 , Pd/C, EtOAc/EtOH (3:1), 25°C , 2 h; (e) TBAF, THF, 25°C , 1 h, 88% for 2 steps; (f) **10**, BEMP, CH_2Cl_2 , 25°C , 18 h, 66%; (g) (polystyrylmethyl)trimethylammonium borohydride, MeOH, 25°C 48 h, 75%; (h) **10**, BEMP, CH_2Cl_2 , 25°C , 20 h, 92%; (i) (polystyrylmethyl)trimethylammonium borohydride, MeOH, 25°C , 30 h, 86%.