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Synthesis and biological evaluation of kresoxim-methyl analogues as novel inhibitors of hypoxia-inducible factor (HIF)-1 accumulation in cancer cells

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ABSTRACT

We designed and synthesized strobilurin analogues as hypoxia-inducible factor (HIF) inhibitors based on the molecular structure of kresoxim-methyl. Biological evaluation in human colorectal cancer HCT116 cells showed that most of the synthesized kresoxim-methyl analogues possessed moderate to potent inhibitory activity against hypoxia-induced HIF-1 transcriptional activation. Three candidates, compounds **11b**, **11c**, and **11d** were identified as potent inhibitors against HIF-1 activation with IC₅₀ values of 0.60–0.94 μM. Under hypoxic condition, compounds **11b**, **11c**, and **11d** increased the intracellular oxygen contents, thereby attenuating the hypoxia-induced accumulation of HIF-1α protein.

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Most solid tumors contain hypoxic regions within the tumor microenvironment and cancer cells adapted to hypoxia are highly resistant to chemotherapy and radiotherapy.¹ Hypoxia-inducible factor (HIF)-1α is a key regulator of the adaptation of cancer cells to hypoxia.² Under normoxic condition, hydroxylation of HIF-1α by prolyl hydroxylase (PHD) allows the recruitment of ubiquitin ligase von Hippel Lindau (VHL), and then HIF-1α undergoes ubiquitin and proteasome-dependent degradation.^{3,4} Under hypoxic condition, stabilized HIF-1α translocates into the nucleus and dimerizes with HIF-1β. The HIF-1α/β heterodimer binds to hypoxia-response element (HRE) and induces transcription of a large number of genes implicated in tumor angiogenesis, glucose metabolism, cell survival and metastasis.² Therefore, HIF-1 is an attractive molecular target for cancer treatment, and various HIF-1 inhibitors have been developed.^{5,6}

Strobilurins, natural products isolated from fungi, are commercial quinone outside inhibitor (Q₀I) fungicides.⁷ The strobilurin mechanism of action is an inhibition of fungal respiration by bind-

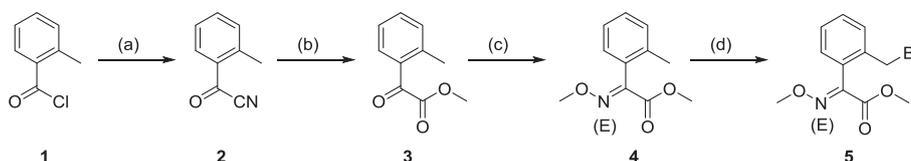
ing to cytochrome b complex at the Q₀ site of complex III in the electron transport chain (ETC) of mitochondria.⁸ Of note, in spite of a broad spectrum of fungicidal activity through the inhibition of respiration, strobilurins induce low toxicity towards mammalian cells.^{8,9} Various biological activities of strobilurin derivatives have been reported including antimalarial,¹⁰ antimicrobial,¹¹ and anticancer activity.¹² Until now, several synthetic strobilurins have been developed, and among them, kresoxim-methyl is one of the most widely used strobilurin fungicides.¹³

Our research has been focused on the development of a small molecule-based HIF-1 inhibitor having an inhibitory activity against mitochondrial respiration. From the results of our previous studies, the inhibition of mitochondrial respiration could lead to the degradation of the HIF-1α protein in cancer cells,^{14–16} indicating that regulation of mitochondria respiration is a promising approach for treatment of hypoxic cancer. In this study, we designed and synthesized kresoxim-methyl analogues as inhibitors of HIF-1α accumulation, and evaluated their HIF inhibitory activity and anti-proliferative activity in cancer cells under hypoxic condition.

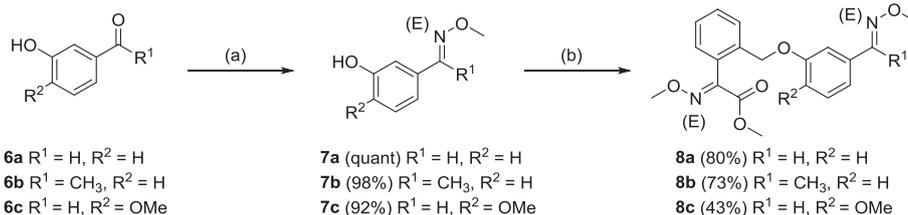
Chemical synthesis of a precursor **5**, (2-bromomethylphenyl) methoxyiminoacetic acid methyl ester, leading to kresoxim-methyl analogues was made using a well-known literature

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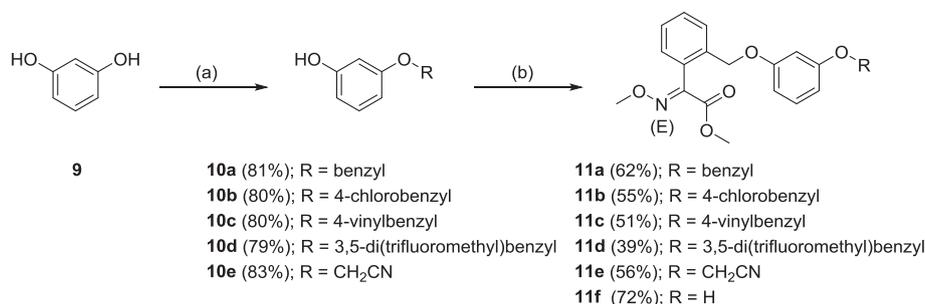
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Scheme 1. Reagents and conditions: (a) NaCN, Aliquat 336, DCM, H₂O, rt, 2 h; (b) 85% H₂SO₄, NaBr, rt, 2 h; MeOH, 65 °C, 2 h; (c) MeONH₂·HCl, MeOH, 65 °C, 12 h; (d) NBS, AIBN, CCl₄, 80 °C, 12 h, 70%.



Scheme 2. Reagents and conditions: (a) *o*-methoxyamine hydrochloride, MeOH, 65 °C, 12 h; (b) **5**, K₂CO₃, acetone, 60 °C, 12 h.



Scheme 3. Reagents and conditions: (a) R-Br, K₂CO₃, acetone, 60 °C, 12 h; (b) **5**, K₂CO₃, acetone, 60 °C, 12 h.

procedure.¹⁷ In these sequences, commercially available *o*-toluoyl chloride was converted to **5** via a four-step route on a multigram scale without column chromatography (Scheme 1). Cyanation of *o*-toluoyl chloride **1** with sodium cyanide and Aliquat 336 in dichloromethane and water (1:1 v/v) quantitatively yielded benzoyl cyanide **2**, which was converted to keto ester **3** by treatment with a methanolic solution of sodium bromide and sulfuric acid. Reaction of **3** with *o*-methylhydroxylamine hydrochloride gave the corresponding imine in a mixture of *E/Z* = 85:15, and following recrystallization from dichloromethane and hexane to afford (*E*)-methyl 2-methoxyimino-2-*o*-tolylacetate **4**. The benzylic radical bromination of **4** using the Wohl–Ziegler procedure with *N*-bromosuccinimide (NBS) yielded **5** in a good yield.

Reaction of phenolic carbonyl compounds **6** with *o*-methoxyamine hydrochloride in methanol gave their corresponding oximes **7** in good yields and resorcinol was converted into its benzylic derivatives **10** in modest to good yields. Finally oximes **7**, resorcinol (**9**) and its benzylic derivatives **10** were reacted with **5** in the presence of potassium carbonate in acetone to afford kresoxim-methyl analogues **8** and **11**, respectively, in modest yields (Schemes 2 and 3).

We next evaluated the biological activity of the synthesized kresoxim-methyl analogues. First, we examined the effects on hypoxia-induced transcriptional activation of HIF-1 using a cell-based HRE reporter gene assay in human colorectal cancer HCT116 cells; the results are shown in Table 1. Under hypoxic conditions, 2-hydroxy compound (**11f**) showed moderate inhibitory activity against HIF-1 activation. Replacement of the hydroxyl group to methyl oxime significantly increased the HIF-1 inhibitory activity (**8a** and **8b**). However, introduction of methoxy group into R¹ position (**8c**) resulted in reduction of the inhibitory activity. Furthermore, the addition of benzyloxy substituents on R² position (**11a–11d**) also showed significant inhibitory activity. Among

Table 1

Effects of kresoxim-methyl analogues on the hypoxia-induced HIF transcriptional activation and cancer cell growth.

Cmpd	R ¹	R ²	HRE-Luc ^a IC ₅₀ (μM) ^b
11f	H	OH	9.16 ± 0.95
8a	H		3.40 ± 0.20
8b	H		4.52 ± 0.15
8c	OCH ₃		14.88 ± 0.19
11a	H		1.12 ± 0.57
11b	H		0.60 ± 0.25
11c	H		0.87 ± 0.28
11d	H		0.94 ± 0.15
11e	H		>20

^a HIF transcriptional activity was determined using HCT116 cells stably transfected with HRE-Luc.

^b The drug concentrations required to inhibit HIF activation by 50% (IC₅₀) was determined from the semilogarithmic dose-response plots, and the results are means ± SD of triplicate samples.

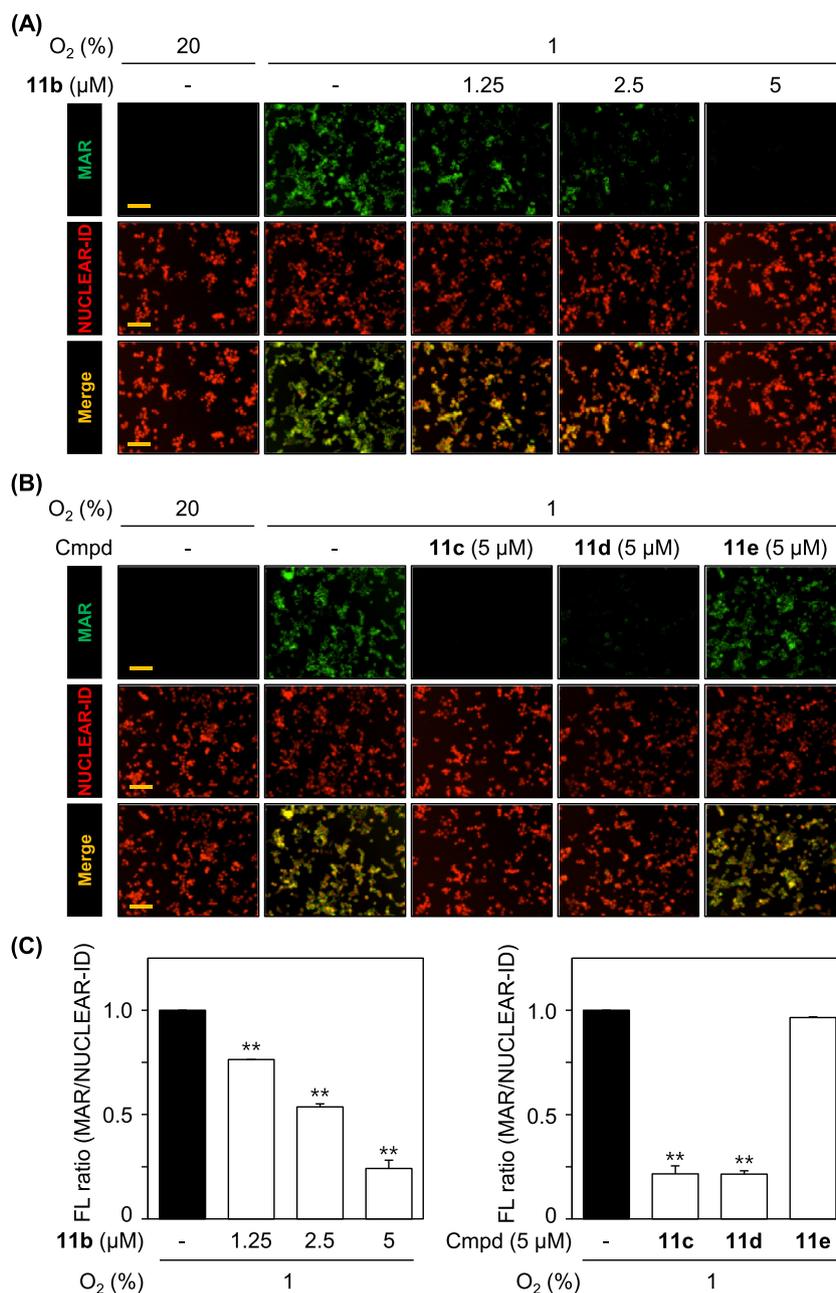


Fig. 1. Effects of kresoxim-methyl analogues on intracellular oxygen tension. HCT116 cells were treated with the indicated concentration of compound **11b** (A) or 5 μM compounds **11c**, **11d**, and **11e** (B) for 6 h under hypoxic conditions. The intracellular oxygen tension was detected by hypoxia-sensitive probe MAR (0.5 μM). Nuclei were stained with NUCLEAR-ID Red DNA stain. The scale bar indicates 100 μm. (C) The fluorescence intensity ratios of MAR probe to NUCLEAR-ID are shown by vertical bars. Statistical significance: ***P* < 0.01, compared with hypoxia control.

them, compound **11b** containing 4-chloro-benzyloxy exerted the most potent inhibitory activity with an IC₅₀ value of 0.6 μM. However, cyanide (**11e**) did not show any influence on hypoxia-induced HIF activation.

To assess ability of the synthesized compounds to regulate intracellular oxygen, we determined the oxygen level in cancer cells using a hypoxia-sensitive fluorescence probe, mono azo rhodamine (MAR).¹⁸ The results are shown in Fig. 1. Under normoxia, the MAR probe is non-fluorescent, but it turns fluorescent due to the reduction of the azo group when exposed to hypoxic conditions. Effects of kresoxim-methyl analogues on the change of intracellular oxygen levels were determined. As shown in Fig. 1A, compound **11b** at 1–5 μM attenuated the reduction of the MAR probe, indicating that the intracellular oxygen level increased in a concentration-dependent manner. In addition, compounds **11c**

and **11d** at 5 μM potentially enhanced the oxygen level, but compound **11e** at 5 μM did not (Fig. 1B). The fluorescent intensity ratio of MAR probe to NUCLEAR-ID is presented in Fig. 1C. Furthermore, other kresoxim-methyl analogues at 5 μM also regulated the intracellular oxygen levels in parallel with the HIF inhibition (Supplementary Fig. S1). These results indicate that the inhibitory effect of HIF activation by kresoxim-methyl analogues is mediated by regulation of intracellular oxygen tension.

We next explored whether the increased oxygen by kresoxim-methyl analogues lead to degradation of the HIF-1α protein in hypoxic cells. As shown in Fig. 2, incubation of HCT116 cells under hypoxia for 6 h significantly induced accumulation of HIF-1α protein. Under these conditions, treatment with compounds **11b**, **11c**, and **11d** at 1 to 5 μM suppressed the hypoxia-induced HIF-1α accumulation in a concentration-dependent manner, while compound

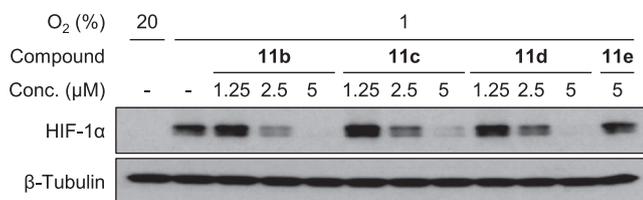


Fig. 2. Effects of kresoxim-methyl analogues on the hypoxia-induced accumulation of HIF-1 α protein. HCT116 cells were incubated for 6 h in the presence of each compound. The protein levels of HIF-1 α and β -tubulin were detected by immunoblot analysis with specific antibodies.

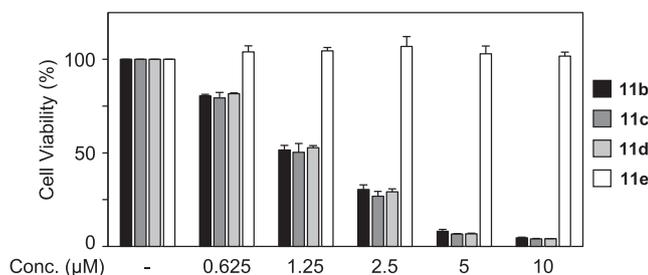


Fig. 3. Effects of kresoxim-methyl analogues on cancer cell viability. HCT116 cells were incubated for 72 h in the presence of each kresoxim-methyl analogue. The cell viability was determined by a methylene blue assay.

11e did not have an effect at 5 μ M. These results indicate that the enhancement of intracellular oxygen tension by kresoxim-methyl analogues promotes the ubiquitin-dependent proteasomal degradation of HIF-1 α .

Next, the effects of kresoxim-methyl analogues on cell growth were examined in HCT116 cells. As shown in Fig. 3, compounds **11b**, **11c**, and **11d** induced potent inhibitory activity on cell growth and their IC₅₀ values were 1.62, 1.26, and 1.36 μ M, respectively, whereas compound **11e** did not exert any effect on cancer cell growth. Furthermore, these compounds showed a similar activity under hypoxic condition (Supplementary Fig. S2).

In conclusion, we designed and synthesized kresoxim-methyl analogues that act as inhibitors of the hypoxia-mediated HIF activation. Biological experiments demonstrated that compounds **11b**, **11c**, and **11d** potently inhibited HIF activation and cell proliferation in HCT116 human colorectal cancer cells. These compounds suppressed the hypoxia-induced accumulation of HIF-1 α protein by increasing the intracellular oxygen level. Taken together, our results from this study could provide an impetus to further develop kresoxim-methyl analogues as anticancer agents.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2017.05.024>.

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