Accepted Manuscript

Synthesis and biological evaluation of 1,2-dithiol-3-thiones and pyrrolo[1,2-*a*]pyrazines as novel hypoxia inducible factor-1 (HIF-1) inhibitor

Young Hun Lee, Jung Min Lee, Sang Geon Kim, Yong Sup Lee

PII: S0968-0896(16)30297-8
DOI: http://dx.doi.org/10.1016/j.bmc.2016.04.054
Reference: BMC 12969

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 12 March 2016
Revised Date: 24 April 2016
Accepted Date: 25 April 2016

Please cite this article as: Lee, Y.H., Lee, J.M., Kim, S.G., Lee, Y.S., Synthesis and biological evaluation of 1,2-dithiol-3-thiones and pyrrolo[1,2-*a*]pyrazines as novel hypoxia inducible factor-1 (HIF-1) inhibitor, *Bioorganic & Medicinal Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.bmc.2016.04.054

Graphical Abstract

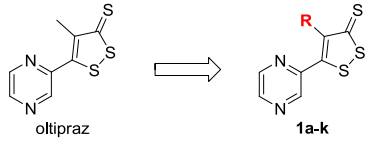
To create your abstract, type over the instructions in the template box below.
Fonts or abstract dimensions should not be changed or altered.

Synthesis and biological evaluation of 1,2-dithiol-3-thiones and pyrrolo[1,2-*a*]pyrazines as novel hypoxia inducible factor-1 (HIF-1) inhibitors

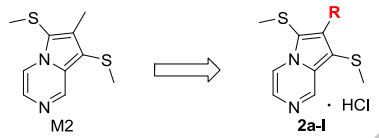
Leave this area blank for abstract info.

Young Hun Lee[a], Jung Min Lee[b], Sang Geon Kim[b], Yong Sup Lee[a,c]*

[a]*Department of Life and Nanopharmaceutical Sciences, Kyung Hee University, Seoul 02447, Republic of Korea*
[b]*Department of Pharmacy, College of Pharmacy, Seoul National University, Seoul 08826, Republic of Korea*
[c]*Medicinal Chemistry Laboratory, Department of Pharmacy, College of Pharmacy, Kyung Hee University, Seoul 02447, Republic of Korea*



	% inhibition at 30 μM		
	HIF-1α	VEGF	GLUT-1
oltipraz (R=Me)	48%	36%	59%
R = propyl	76%	70%	86%



	% inhibition at 10 μM		
	HIF-1α	VEGF	GLUT-1
M2 (R=Me)	37%	no inhibition	14%
R = propyl	90%	69%	83%
R = benzyl	81%	77%	92%

Bioorganic & Medicinal Chemistry
journal homepage: www.elsevier.com

Synthesis and biological evaluation of 1,2-dithiol-3-thiones and pyrrolo[1,2-*a*]pyrazines as novel hypoxia inducible factor-1 (HIF-1) inhibitor

Young Hun Lee[a], Jung Min Lee[b], Sang Geon Kim[b] and Yong Sup Lee[a,c]*

[a]*Department of Life and Nanopharmaceutical Sciences, Kyung Hee University, Seoul 02447, Republic of Korea*
[b]*Department of Pharmacy, College of Pharmacy, Seoul National University, Seoul 08826, Republic of Korea*
[c]*Medicinal Chemistry Laboratory, Department of Pharmacy, College of Pharmacy, Kyung Hee University, Seoul 02447, Republic of Korea*

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Hypoxia inducible factor-1
VEGF
GLUT-1
oltipraz
pyrrolopyrazine

ABSTRACT

Hypoxia-inducible factor-1 (HIF-1) is a key transcription factor which is strongly associated with tumor survival, progression, and therapeutic resistance. Accordingly, it has been suggested that the inhibition of the HIF-1 pathway can suppress tumor, and it has become an important therapeutic target. In present study, oltipraz, its metabolite M2, and their derivatives were synthesized and evaluated as HIF-1α inhibitors. Among the synthesized, benzyl-substituted pyrrolo[1,2-a]pyrazine **2g** most potently inhibited HIF-1α protein accumulation (81% at 10 μM) and VEGF, GLUT-1 transcription (77% and 92% at 10 μM, respectively).

1. Introduction

Inadequate vascularization associated with fast proliferating solid tumors limits efficient oxygen supply and renders the tumor hypoxic, which eventually leads to tumor necrosis. In hypoxic condition, however, many of cancer cells promote appropriate responses such as anaerobic metabolism and increased oxygen delivery so that they can survive and even proliferate in a hypoxic environment. These processes hinder the effectiveness of chemotherapy and radiotherapy, and they also contribute to aggressive tumor behavior.[1,2]

Hypoxia-inducible factor-1 (HIF-1) is a major modulator expressed in many tumor cells and responsible for orchestrating diverse cellular responses such as angiogenesis and glycolysis that help cancer cells adapt to hypoxic conditions.[3] At normal oxygen levels, HIF-1α is continually degraded by the pVHL (von Hippel-Lindau protein)-mediated ubiquitination and proteasomal degradation and the degradation pathway requires O_2. Therefore, under hypoxic conditions, HIF-1α rapidly accumulates in the cell and dimerizes with HIF-1β to form the active transcription factor HIF-1.[4,5] HIF-1 binds to hypoxia-responsive elements (HRE) with co-activator p300 and CBP. The binding activates the transcription of variety of genes involved in angiogenesis, glycolysis, growth factor signaling, tumor invasion, and metastasis.[6] Moreover, many cancer cells induce HIF-1 pathway O_2-independently via enhancing *de novo* synthesis of HIF-1α protein using a wide range of growth-promoting stimuli and oncogenic signals for their survival and proliferation.[7]

HIF-1 have been evaluated as a cancer therapeutic target via a variety of approaches. Clinically, overexpression of HIF-1α has been found in many cancer cells,[8–10] and this is associated with treatment failure and increased patient mortality.[11–14] The inhibition of the HIF-1 pathway significantly inhibits tumor growth in animal models.[15] Accordingly, HIF-1 represents an attractive molecular target for the development of novel anticancer agents.[16]

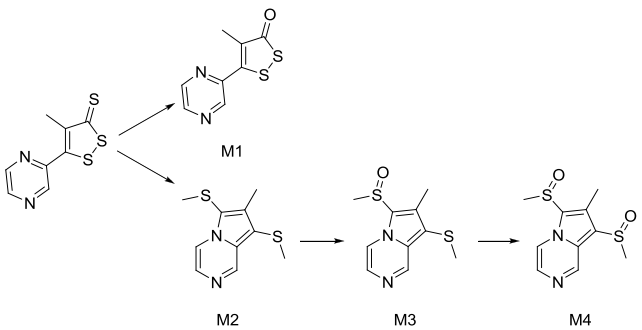


Figure 1. Chemical structures of oltipraz metabolites M1–4

* Corresponding author. Tel.: +82-2-961-0370; e-mail: kyslee@khu.ac.kr

A growing number of studies have reported that a series of compounds, such as YC-1, rapamycin, PX-478, and 17-AAG, inhibit HIF-1α pathway in cellular and animal models.[17–20] However, because of their toxicity and other adverse effects, current pharmacologic interventions in HIF-1α activity are limited and expected to be advanced by tailor-made drugs.

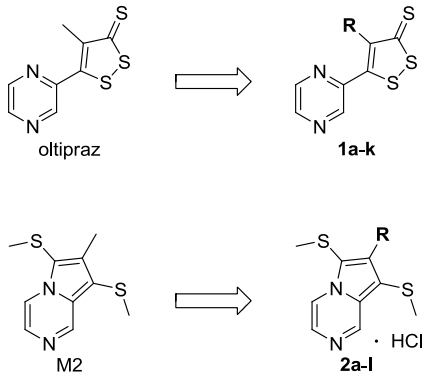


Figure 2. Structures of oltipraz, M2, and their derivatives **1a–k**, **2a–l**.

Oltipraz is a synthetic dithiolethione (5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione) that is structurally similar to the dithiolethiones found in cruciferous vegetables. It originally was marketed by Rhone-Poulenc (Vitry-sur-Seine, France) for the treatment of schistosomiasis, and extensively evaluated as a chemopreventive agent in the 1990s.[6,21]

Recent studies revealed that oltipraz inhibits HIF-1 pathway by affecting both synthesis and degradation of HIF-1α. Oltipraz was found to inhibit HIF-1α synthesis by suppressing mammalian target of rapamycin (mTOR) and p70 ribosomal S6 kinase-1 (S6K1) pathway and promote degradation by facilitating ubiquitylation.[22] Separate pharmacokinetic studies revealed that oltipraz is metabolized by the two major pathways: first, oxidative desulfuration of the thione to yield M1, which does not seem to be metabolized further; and second, desulfuration, methylation, and molecular rearrangement to yield M2 (7-methyl-6,8-bis(methylthio)pyrrolo[1,2-*a*]pyrazine), which can be metabolized to other oxidized forms, M3 and M4 **(Figure 1)**.[23] M2 was also reported to suppress HIF-1 pathway by inhibiting *de novo* synthesis of HIF-1α via induction of microRNAs 199a-5p and 20a indicating that M2 has distinct mechanism of HIF-1α inhibition in comparison to oltipraz.[24] Such a mechanism differs from other reported HIF-1 inhibitors and may lead to significant insight into novel approaches to tumor suppression.

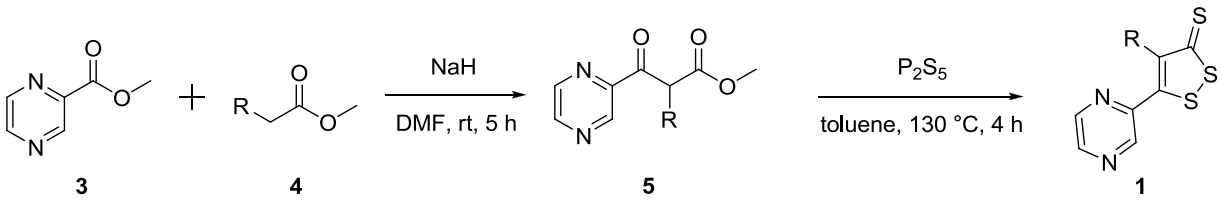
In the present study, we synthesized new 4-substituted 1,2-dithiol-3-thiones **1a–k** (oltipraz derivatives) and 7-substituted pyrrolo[1,2-*a*]pyrazines **2a–l** (M2 derivatives), in order to study the structure-activity relationships, and thereby, to provide new leads possessing enhanced HIF-1 inhibitory activity in comparison to oltipraz and M2. We introduced various alkyl chain to determine the effects of the length of alkyl group, and various phenyl, benzyl, phenethyl group to determine the effects of the additional aromatic ring linked to the parent compound. All the pyrrolopyrazine compounds **2a–l** were synthesized as HCl salt to increase stability and water solubility. **(Figure 2)**

2. Results and discussion

2.1. Chemistry

The syntheses of 1,2-dithiol-3-thione derivatives **1a–k** were accomplished using the procedures shown in Scheme 1. Mixed Claisen condensation of methyl pyrazine-2-carboxylate **3** with various ester compounds **4a–k** yielded the 2-substituted 3-oxoester intermediates **5a–k**. Since the yields of thionation reactions are largely affected by the purity of 3-oxoester intermediates, compounds **5a–k** were purified by recrystallization in EtOAc/n-Hexane. The 1,2-dithiol-3-thione compounds are usually synthesized from 3-oxoesters using thionation reaction, which suffers from low yield.[25] There are many efforts to improve the thionation reaction, but the thionation reaction of 3-oxoesters which have pyrazinyl moiety at C-3 still shows low yield. Among the reported procedure, we chose the recent and improved procedure reported by Curphey *et al*.[26] and the modification of the procedure was used to synthesize 1,2-dithiol-3-thione compounds **1a–k**. Compounds **5a–k** were reacted with P_4S_{10} in refluxing toluene and recrystallized in acetonitrile to give **1a–k** in acceptable yields.

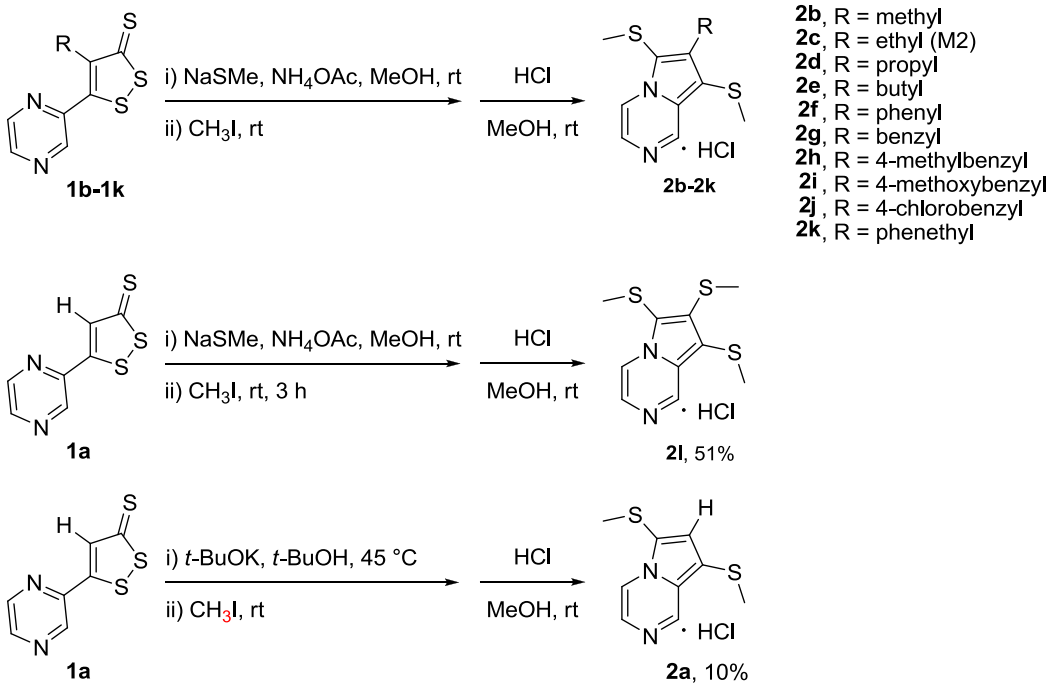
The pyrrolo[1,2-*a*]pyrazine derivatives **2a–l** were synthesized by using the modification of Largeron's method. (Scheme 2) Lageron *et al*. reported that M2 could be synthesized from oltipraz by rearrangement using sodium thiomethoxide (NaSMe).[27] The key features of the rearrangement are nucleophilic attack of MeS^- at S-2 position and intramolecular ring closure with elimination of molecular sulfur. Compounds **1b–k** were reacted with NaSMe to undergo rearrangement that formulates pyrrolopyrazine structure, and then reacted with CH_3I



5a, R = H
5b, R = methyl
5c, R = ethyl
5d, R = propyl
5e, R = butyl
5f, R = phenyl
5g, R = benzyl
5h, R = 4-methylbenzyl
5i, R = 4-methoxybenzyl
5j, R = 4-chlorobenzyl
5k, R = phenethyl

1a, R = H
1b, R = methyl (oltipraz)
1c, R = ethyl
1d, R = propyl
1e, R = butyl
1f, R = phenyl
1g, R = benzyl
1h, R = 4-methylbenzyl
1i, R = 4-methoxybenzyl
1j, R = 4-chlorobenzyl
1k, R = phenethyl

Scheme 1. Synthesis of compounds **1a–k**



Scheme 2. Synthesis of compounds **2a–l**

to yield compounds **2b**–**k** as free base form. The resulting compounds were dissolved in 1.25 M HCl methanol solution, stirred for 1 h and then precipitated in MeOH/Et_2O solution to yield compounds **2b**–**k** as HCl salt. However, reaction of **1a** with NaSMe followed by methylation using CH_3I yielded 7-methylthio substituted compound **2l** instead of **2a**. It was supposed that the thiomethoxide attacked C-4 instead of S-2 and the following ring closure resulted the compound **2l**. Finally, compound **2a** could be synthesized using *t*-BuOK as nucleophile in *t*-BuOH.

2.2. Inhibitory effects of 1a–k and 2a–l on insulin-induced HIF-1α accumulation

It has been known that insulin decreases both synthesis and stability of HIF-1α. Insulin affects the stability of the HIF-1α protein by generating ROS, in particular H_2O_2.[28,29] H_2O_2 inactivates proline hydroxylase-domain enzymes, which initiate ubiquitylation and degradation, thereby stabilizing HIF-1α.[29] Insulin also promotes HIF1A mRNA translation and thus increases *de novo* synthesis of HIF-1α through the mTOR/ S6K1 pathway.[30] Accordingly, the HIF-1α protein levels were determined using Western blot assay to evaluate the effects of the synthesized compounds **1a**–**k** and **2a**–**l** on insulin induced HIF-1α accumulation. The effects on HIF-1α expression were monitored at proper concentration (**1a**–**k**: 30 μM, **2a**–**l**: 10 μM) in which most of the compounds showed effective HIF-1α inhibition and did not show significant cytotoxicities. Few compounds (**1a**, **2h**–**j**) exhibited severe cytotoxicity at the assay condition and their effects on HIF-1α accumulation were not determined. Topotecan and YC-1 were used as positive control and their effects were determined at 3 μM in which the compounds showed effective inhibitory activities without significant cytotoxicities. The results are listed in **Figure 3**.

As shown in **Figure 3**, insulin induced HIF-1α accumulations were potently inhibited by several dithiolethiones (**1d**: 76%; **1e**: 80%) and pyrrolopyrazines (**2c**: 86%; **2d**: 90%; **2g**: 81%) at 30

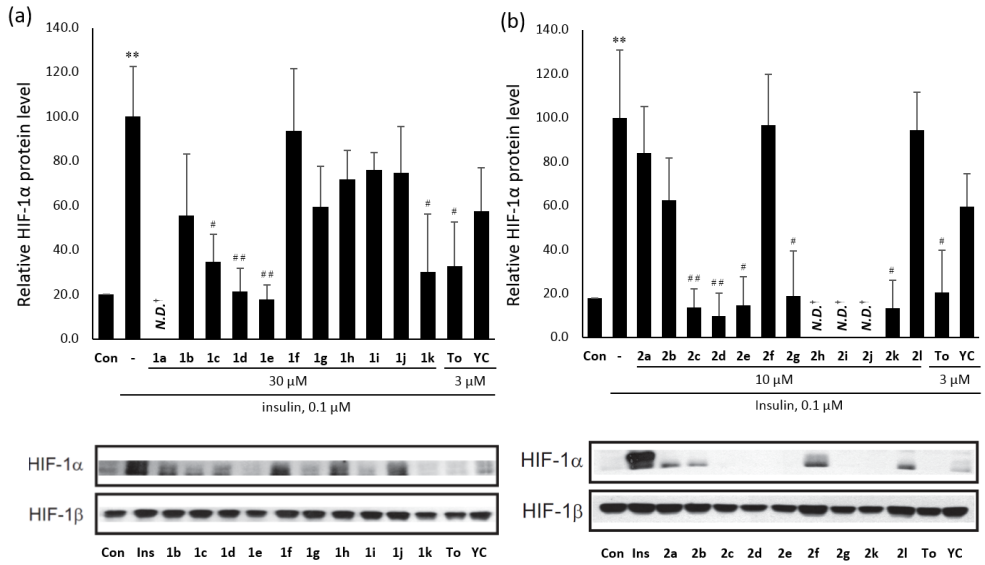


Figure 3. Inhibitory effects of **1a**–**k** (**Figure 3a**) and **2a**–**l** (**Figure 3b**) on insulin-induced HIF-1α accumulation assessed by protein immunoblot (Western blot) analysis. The values above the figures represent relative density of the bands normalized to HIF-1β. The values are mean ± S.E.M. from three independent experiments. **significantly different from vehicle-treated control ($P < 0.01$); #significantly different from insulin-treated control ($P < 0.05$); ##significantly different from insulin-treated control ($P < 0.01$); †not determined due to cytotoxicity; To, topotecan (positive control); YC, YC-1 (positive control)

μM, 10 μM respectively. The inhibitory activities of these compounds were higher than those of their parent compounds (**1b**: 48%; **2b**: 37%). Generally, pyrrolopyrazine derivatives showed similar or higher inhibitory activities at lower concentration in comparison to dithiolethione compounds. It is noteworthy that the analogues with the longer alkyl moiety showed the more potent inhibitory activities. The inhibitory activities of phenyl substituted analogues **1f** (8%) and **2f** (3%) dramatically diminished. This is likely due to an inappropriate positioning of phenyl ring which induces undesired interaction with target protein. A comparison of the phenyl moiety of **1f** (8%), the benzyl moiety of **1g** (41%), and the phenethyl moiety of **1k** (65%) showed that additional aromatic substituents with appropriate alkyl linker could enhance HIF-1 inhibitory activities. The similar trend can be seen with the pyrrolopyrazine derivatives (**2f**: 3%; **2g**: 81%; **2k**: 87%). The 7-methylthio substitution (**2l**: 6%) resulted in a significant decrease in HIF-1α inhibition.

2.3. Inhibitory effects of 1a–k and 2a–l on insulin-induced HIF-1 target gene transcription

A subset of these compounds was analyzed by RT-PCR to confirm that inhibition of HIF-1α results in decreased expression of its target genes. The effects of selected compounds on the mRNA expression of VEGF and GLUT-1 were determined, both of which are well-known HIF-1 target genes and associated with the aggressive tumor phenotype.[3] Generally, the RT-PCR analysis results are well correlated with the Western blot data (**Figure 4**). Compounds with long alkyl substituents (**1d**, **2d**) potently inhibited target gene expression and phenyl substituted compounds (**1f**, **2f**) showed weak inhibition. The propyl-substituted **1d** exhibited the most potent inhibition (VEGF: 70%, GLUT-1: 86%, at 30 μM) among the dithiolethione derivatives, and benzyl-substituted **2g** showed the most potent inhibition (VEGF: 77%, GLUT-1: 92%, at 10 μM) among the pyrrolopyrazine derivatives. The inhibitory activities of these two compounds were markedly increased in comparison to their parent compound **1b** (VEGF: 36%, GLUT-1: 59%, at 30 μM) and **2b** (VEGF: no inhibition, GLUT-1: 14%, at 10 μM). These results indicate that the synthesized compounds can inhibit target gene expression as well as HIF-1α accumulation showing the promising anticancer activities of the compounds.

3. Conclusion

In summary, we have synthesized 11 dithiolethione compounds and 12 pyrrolopyrazine compounds based on oltipraz and its metabolite M2, respectively. These compounds were evaluated for their inhibitory activities on insulin induced HIF-1α accumulation and HIF-1 target gene VEGF and GLUT-1 expressions. The introduction of long alkyl moiety enhanced HIF-1α inhibition in both dithiolethione and pyrrolopyrazine derivatives. Benzyl and phenethyl substitutions also increased inhibitory activities indicating that additional aromatic substituents with proper alkyl linker could improve HIF-1α

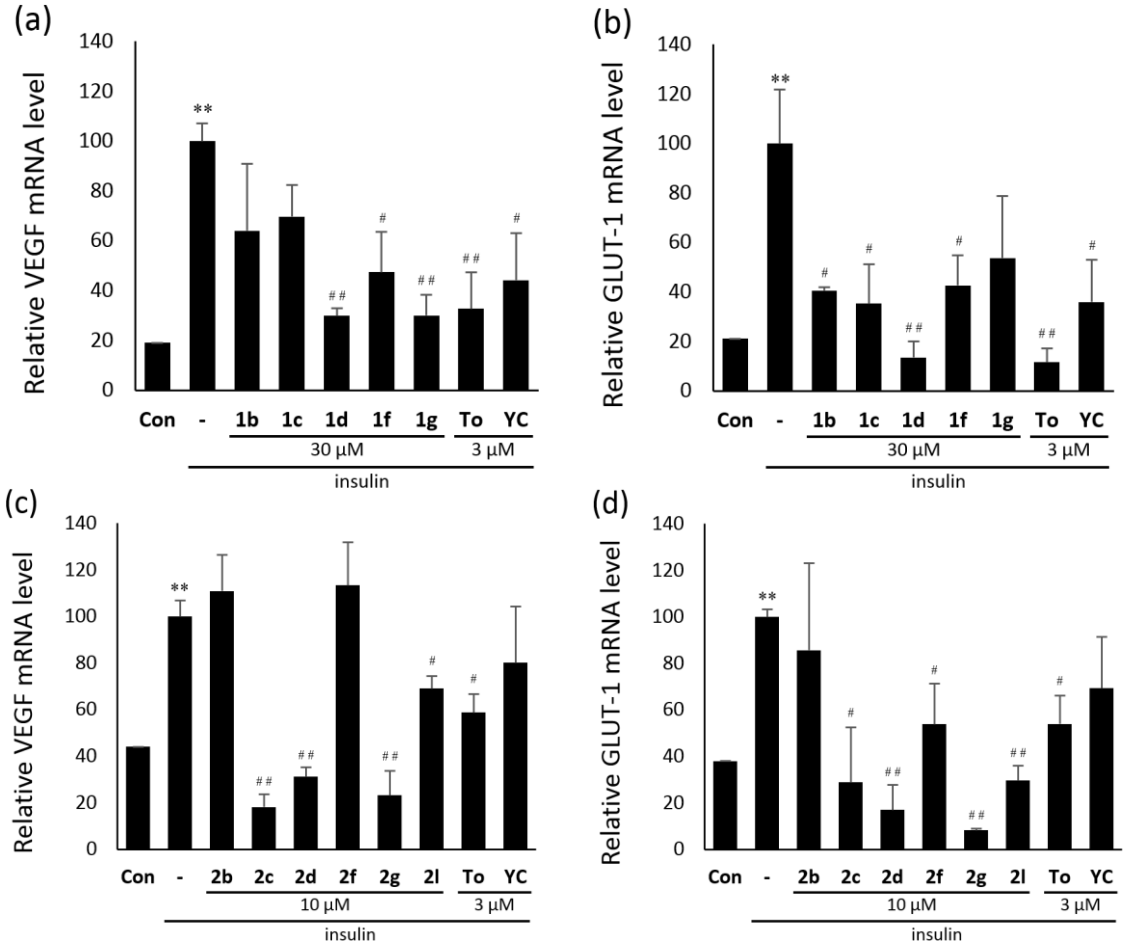


Figure 4. Inhibitory effects of the selected subset of compounds on insulin-induced HIF-1 target gene expression. The values are mean ± S.E.M. from three independent experiments. **significantly different from vehicle-treated control ($P < 0.01$); #significantly different from insulin-treated control ($P < 0.05$); ##significantly different from insulin-treated control ($P < 0.01$); To, topotecan (positive control); YC, YC-1 (positive control)

inhibition. Among the synthesized compounds, compound **2g** containing benzyl moiety at 7-position most potently inhibited HIF-1α accumulation (81%) and their target gene expressions (VEGF: 77%, GLUT-1: 92%) at 10 μM without severe cytotoxicity showing marked enhancement of inhibitory activity in comparison to those of M2 (HIF-1α: 37%, VEGF: no inhibition, GLUT-1: 14%). These results provided an insight into structure-activity relationship of 4-substituted dithiolethiones and 7-substituted pyrrolopyrazines.

4. Experimental.

4.1. Instrumentation and reagents

Nuclear magnetic resonance (NMR) spectral analyses were performed using a Brucker Avance 400 spectrometer operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR spectra. Chemical shifts (δ) are reported in ppm, downfield from internal tetramethylsilane (TMS) standard. High resolution mass spectra (HRMS) were recorded on a Jeol accuTOF (JMS-T100TD) equipped with a DART (direct analysis in real time) ion source from Ionsense (Tokyo, Japan) in the positive modes. Analytical thin layer chromatography (TLC) was carried out using precoated silica gel (E. Merck Kiesegel 60F_{254}, layer thickness 0.25 mm) and flash column chromatography was performed with using Merck Kiesegel 60 Art 9385 (230–400 mesh). All solvents, chemicals and reagents were purchased from Sigma-Aldrich or Tokyo chemical industry (TCI), and used without further purification. Antibodies specifically directed against HIF-1α and HIF-1β were purchased from Becton-Dickinson Biosciences, Anti-ubiquitin antibody was obtained from Sigma-Aldrich chemical. Antibodies recognizing S6K1, p-S6K1, lamin A/C and HSP70 were purchased from Cell Signaling Technology.

4.2. Synthesis

4.2.1 Methyl pyrazine-2-carboxylate

Pyrazine-2-carboxylic acid (5 g, 40.3 mmol) was dissolved in MeOH (150 ml), and a few drops of H_2SO_4 were added. The resulting reaction mixture was refluxed for 2 h. Methanol was evaporated and the resulting reaction mixture was extracted with EtOAc, washed with saturated $NaHCO_3$ solution, then with brine and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* to give the methyl pyrazine-2-carboxylate (5.5 g, yield 98%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.32 (d, 1H, J = 1.5 Hz), 8.78 (d, 1H, J = 2.4 Hz), 8.73 (dd, 1H, J = 2.4, 1.5 Hz), 4.04 (s, 3H).

4.2.2 Methyl 3-oxo-3-(pyrazin-2-yl)propanoate (5a)

To a mixture of NaH (60% in mineral oil, 0.68 g, 28.2 mmol), methyl pyrazine-2-carboxylate (3 g, 21.7 mmol) and dry DMF (30 mL), methyl acetate (2.09 g, 28.2 mmol) was added dropwise under N_2 atmosphere. After being stirred at rt for 5 h, the reaction mixture was concentrated *in vacuo*, and the residue treated with saturated aqueous $NaHCO_3$ solution followed by extraction with EtOAc. The combined organic phase were dried with $MgSO_4$, filtered, and concentrated *in vacuo*. Column chromatographic purification (*n*-hexane / EtOAc = 8 : 1) yielded **5a** as white solid (1.95 g, 50%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ keto form: 9.28 (d, 1H, J = 1.5 Hz), 8.80 (d, 1H, J = 2.4 Hz), 8.66 (overlapped, 1H), 4.18 (s, 2H), 3.74 (s, 3H); enol form: 12.30 (s, 1H), 9.15 (d, 1H, J = 1.5 Hz), 8.66 (overlapped, 1H), 8.60 (dd, 1H, J = 2.3, 1.5 Hz), 6.34 (s, 1H), 3.85 (s, 3H).

4.2.3. Methyl 2-methyl-3-oxo-3-(pyrazin-2-yl)propanoate (5b)

The compound **5b** was prepared from methyl pyrazine-2-carboxylate and methyl propionate using the procedure described for **5a**. Yield 79%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.28 (d, 1H, J = 1.4 Hz), 8.78 (d, 1H, J = 2.4 Hz), 8.64 (dd, 1H, J = 2.4, 1.4 Hz), 4.68 (q, 1H, J = 7.1 Hz), 3.68 (s, 3H), 1.52 (d, 3H, J = 7.1 Hz).

4.2.4. Methyl 2-(pyrazine-2-carbonyl)butanoate (5c)

The compound **5c** was prepared from methyl pyrazine-2-carboxylate and methyl butyrate using the procedure described for **5a**. Yield 64%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.28 (d, 1H, J = 1.4 Hz), 8.78 (d, 1H, J = 2.4 Hz), 8.65 (dd, 1H, J = 2.4, 1.4 Hz), 4.57 (t, 1H, J = 7.3 Hz), 3.67 (s, 3H), 2.06 (m, 2H), 1.02 (t, 3H, J = 7.3 Hz).

4.2.5. Methyl 2-(pyrazine-2-carbonyl)pentanoate (5d)

The compound **5d** was prepared from methyl pyrazine-2-carboxylate and methyl pentanoate using the procedure described for **5a**. Yield 75%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.27 (d, 1H, J = 1.5 Hz), 8.78 (d, 1H, J = 2.4 Hz), 8.65 (dd, 1H, J = 2.4, 1.5 Hz), 4.66 (t, 1H, J = 7.1 Hz), 3.67 (s, 3H), 2.00 (m, 2H), 1.41 (m, 2H), 0.95 (t, 3H, J = 7.3 Hz).

4.2.6. Methyl 2-(pyrazine-2-carbonyl)hexanoate (5e)

The compound **5e** was prepared from methyl pyrazine-2-carboxylate and methyl pentanoate using the procedure described for **5a**. Yield 49%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.27 (d, 1H, J = 1.5 Hz), 8.78 (d, 1H, J = 2.4 Hz), 8.65 (dd, 1H, J = 2.4, 1.5 Hz), 4.64 (t, 1H, J = 7.1 Hz), 3.67 (s, 3H), 2.06–1.97 (m, 2H), 1.41–1.29 (m, 4H), 0.93–0.86 (m, 3H).

4.2.7. Methyl 3-oxo-2-phenyl-3-(pyrazin-2-yl)propanoate (5f)

The compound **5f** was prepared from methyl pyrazine-2-carboxylate and methyl 2-phenylacetate using the procedure described for **5a**. Yield 41%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.28 (d, 1H, J = 1.4 Hz), 8.76 (d, 1H, J = 2.4 Hz), 8.65 (dd, 1H, J = 2.4, 1.4 Hz), 7.45–7.30 (m, 5H), 6.11 (s, 1H), 3.74 (s, 3H).

4.2.8. Methyl 2-benzyl-3-oxo-3-(pyrazin-2-yl)propanoate (5g)

The compound **5g** was prepared from methyl pyrazine-2-carboxylate and methyl 3-phenylpropanoate using the procedure described for **5a**. Yield 60%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.22 (d, 1H, J = 1.5 Hz), 8.75 (d, 1H, J = 2.4 Hz), 8.62 (dd, 1H, J = 2.4, 1.5 Hz), 7.26–7.14 (m, 5H), 5.10 (dd, 1H, J = 7.8, 6.8 Hz), 3.64 (s, 3H), 3.37 (dd, 1H, J = 13.7, 7.8 Hz), 3.32 (dd, 1H, J = 13.7, 6.8 Hz).

4.2.9. Methyl 2-(4-methylbenzyl)-3-oxo-3-(pyrazin-2-yl)propanoate (5h)

The compound **5h** was prepared from methyl pyrazine-2-carboxylate and methyl 3-*p*-tolylpropanoate using the procedure described for **5a**. Yield 51%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.23 (d, 1H, J = 1.5 Hz), 8.76 (d, 1H, J = 2.4 Hz), 8.63 (dd, 1H, J = 2.4, 1.5 Hz), 7.17–7.12 (m, 2H), 7.09–7.04 (m, 2H), 5.08 (dd, 1H, J = 7.8, 6.8 Hz), 3.66 (s, 3H), 3.35 (dd, 1H, J = 14.2, 7.8 Hz), 3.30 (dd, 1H, J = 14.2, 6.8 Hz), 2.29 (s, 3H).

4.2.10. Methyl 2-(4-methoxybenzyl)-3-oxo-3-(pyrazin-2-yl)propanoate (5i)

The compound **5i** was prepared from methyl pyrazine-2-carboxylate and methyl 3-(4-methoxyphenyl)propanoate using the procedure described for **5a**. Yield 44%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.21 (d, 1H, J = 1.5 Hz), 8.74 (d, 1H, J = 2.4 Hz), 8.61 (dd, 1H, J = 2.4, 1.5 Hz), 7.17–7.13 (m, 2H), 6.79–6.75 (m, 2H), 5.05 (dd, 1H, J = 7.8, 6.8 Hz), 3.74 (s, 3H), 3.63 (s, 3H), 3.31 (dd, 1H, J = 14.2, 7.8 Hz), 3.26 (dd, 1H, J = 14.2, 6.8 Hz).

4.2.11. Methyl 2-(4-chlorobenzyl)-3-oxo-3-(pyrazin-2-yl)propanoate (5j)

The compound **5j** was prepared from methyl pyrazine-2-carboxylate and methyl 3-(4-chlorophenyl)propanoate using the procedure described for **5a**. Yield 36%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.23 (d, 1H, J = 1.5 Hz), 8.76 (d, 1H, J = 2.4 Hz), 8.62 (dd, 1H, J = 2.4, 1.5 Hz), 7.23–7.16 (m, 4H), 5.06 (dd, 1H, J = 7.8, 6.8 Hz), 3.74 (s, 3H), 3.63 (s, 3H), 3.33 (dd, 1H, J = 14.2, 7.8 Hz), 3.29 (dd, 1H, J = 14.2, 6.8 Hz).

4.2.12. Methyl 4-phenyl-2-(pyrazine-2-carbonyl)butanoate (5k)

The compound **5k** was prepared from methyl pyrazine-2-carboxylate and methyl 4-phenylbutanoate using the procedure described for **5a**. Yield 40%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.24 (s, 1H), 8.76 (s, 1H), 8.62 (s, 1H), 7.29–7.15 (m, 5H), 4.66 (t, 1H, J = 6.9 Hz), 3.67 (s, 3H), 2.83–2.62 (m, 2H), 2.43–2.28 (m, 2H).

4.2.13. 5-(Pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1a)

P_4S_{10} (12 g, 27.0 mmol), toluene (90 ml) and xylene (90 ml) were added to a reactor and heated to a temperature of 120 °C. Compound **5a** (8.1 g, 45.1 mmol) was added to the reactor. The reaction mixture was allowed to proceed under reflux at 135 °C for 4 h and then cooled to 20 °C and filtered through Celite. The solvent was then removed under reduced pressure and the resultant red solid was extracted with CH_2Cl_2. The organic layer was washed with aqueous K_2CO_3 solution, dried over Na_2SO_4, and filtered. The solvent was removed under reduced pressure and the crude solid was dried *in vacuo*. The dried solid was washed with cold EtOAc, and then recrystallized in acetonitrile to give **1a** (0.9 g, 10%) as a red solid. mp 202 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.05 (d, 1H, J = 1.4 Hz), 8.74 (d, 1H, J = 2.4 Hz), 8.66 (dd, 1H, J = 2.4, 1.4 Hz), 7.76 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.7, 168.3, 146.9, 146.1, 144.7, 141.5, 136.0; HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_7H_4N_2S_3$, 212.9609, found, 212.9575.

4.2.14. 4-Methyl-5-(pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1b)

The compound **1b** was prepared from compound **5b** using the procedure described for **1a**. Yield 14%, red solid. mp 165 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.01 (d, 1H, J = 1.2 Hz), 8.76–8.72 (m, 2H), 2.51 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.8, 163.0, 148.0, 145.9, 144.7, 143.6, 142.5, 17.5; HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_8H_6N_2S_3$, 226.9766, found, 226.9714.

4.2.15. 4-Ethyl-5-(pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1c)

The compound **1c** was prepared from compound **5c** using the procedure described for **1a**. Yield 5%, red solid. mp 102 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.10 (d, 1H, J = 1.5 Hz), 8.90 (d, 1H, J = 2.4 Hz), 8.89 (dd, 1H, J = 2.4, 1.5 Hz), 2.87 (q, 2H, J = 7.3 Hz), 1.11 (t, 3H, J = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.9, 163.6, 148.4, 148.0, 146.2, 145.0, 143.4, 24.1, 12.8; HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_9H_8N_2S_3$, 240.9922, found, 240.9879.

4.2.16. 4-Propyl-5-(pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1d)

The compound **1d** was prepared from compound **5d** using the procedure described for **1a**. Yield 7%, red solid. mp 111 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.96 (d, 1H, J = 1.0 Hz), 8.76–8.73 (m, 2H), 2.91 (m, 2H), 1.65 (m, 2H), 1.00 (t, 3H, J = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 217.1, 163.6, 148.1, 147.3, 146.1, 144.9, 143.4, 32.5, 21.7, 14.4; HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_{10}H_{10}N_2S_3$, 255.0079, found, 255.0022.

4.2.17. 4-Butyl-5-(pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1e)

The compound **1e** was prepared from compound **5e** using the procedure described for **1a**. Yield 7%, red solid. mp 67 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.97 (d, 1H, J = 1.0 Hz), 8.76–8.73 (m, 2H), 2.97–2.91 (m, 2H), 1.64–1.54 (m, 2H), 1.48–1.36 (m, 2H), 0.93 (t, 3H, J = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 217.1, 163.4, 148.1, 147.5, 146.1, 144.9, 143.4, 30.3, 30.2, 23.0, 14.0; HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_{11}H_{12}N_2S_3$, 269.0235, found, 269.0169.

4.2.18. 4-Phenyl-5-(pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1f)

The compound **1f** was prepared from compound **5f** using the procedure described for **1a**. Yield 14%, red solid. mp 213 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.62 (dd, 1H, J = 2.4, 1.5 Hz), 8.56 (d, 1H, J = 2.4 Hz), 7.88 (d, 1H, J = 1.5 Hz), 7.55–7.49 (m, 3H), 7.28–7.23 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.3, 165.7, 147.4, 146.3, 145.7, 144.0, 143.4, 134.1, 130.1, 129.8 (2C), 129.7 (2C); HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_{13}H_8N_2S_3$, 288.9922, found, 288.9873.

4.2.19. 4-Benzyl-5-(pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1g)

The compound **1g** was prepared from compound **5g** using the procedure described for **1a**. Yield 16%, red solid. mp 167 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.77 (d, 1H, J = 1.0 Hz), 8.69–8.66 (m, 2H), 7.27–7.06 (m, 5H), 4.41 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.9, 165.8, 147.6, 146.3, 144.9, 144.8, 143.6, 137.3, 129.1 (2C), 128.1 (2C), 126.9, 35.8; HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_{14}H_{10}N_2S_3$, 303.0079, found, 303.0016.

4.2.20. 4-(Methylbenzyl)-5-(pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1h)

The compound **1h** was prepared from compound **5h** using the procedure described for **1a**. Yield 16%, red solid. mp 158 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.78 (d, 1H, J = 1.0 Hz), 8.69–8.66 (m, 2H), 7.08–7.02 (m, 2H), 7.00–6.95 (m, 2H), 4.36 (s, 2H), 2.28 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.8, 165.5, 147.4, 146.1, 144.8, 144.5, 143.4, 136.3, 133.9, 129.5 (2C), 127.7 (2C), 35.2, 21.0; HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_{15}H_{12}N_2S_3$, 317.0235, found, 317.0206.

4.2.21. 4-(Methoxybenzyl)-5-(pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1i)

The compound **1i** was prepared from compound **5i** using the procedure described for **1a**. Yield 13%, red solid. mp 122 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.79 (d, 1H, J = 1.0 Hz), 8.69–8.66 (m, 2H), 7.06–6.96 (m, 2H), 6.84–6.72 (m, 2H), 4.33 (s, 2H), 3.75 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.8, 165.4, 158.3, 147.4, 146.1, 145.0, 144.5, 143.5, 129.0 (2C), 128.9 (2C), 114.3, 55.2, 34.8; HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_{15}H_{12}N_2OS_3$, 333.0185, found, 333.0095.

4.2.22. 4-(Chlorobenzyl)-5-(pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1j)

The compound **1j** was prepared from compound **5j** using the procedure described for **1a**. Yield 15%, red solid. mp 152 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.78 (s, 1H), 8.72 (s, 2H), 7.25–7.21 (m, 2H), 7.08–7.04 (m, 2H), 4.38 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.6, 165.8, 147.5, 146.5, 144.9, 144.5, 143.5, 135.8, 132.8, 129.5 (2C), 129.1 (2C), 35.2; HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_{14}H_9ClN_2S_3$, 336.9689, found, 336.9731.

4.2.23. 4-(Phenethyl)-5-(pyrazin-2-yl)-3*H*-1,2-dithiole-3-thione (1k)

The compound **1k** was prepared from compound **5k** using the procedure described for **1a**. Yield 11%, red solid. mp 68 °C; ^{1}H

NMR ($CDCl_3$, 400 MHz) δ 8.70–8.65 (m, 3H), 7.24–7.13 (m, 3H), 7.11–7.07 (m, 2H), 3.25 (t, 2H, J = 7.7 Hz), 2.93 (t, 2H, J = 7.7 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.5, 163.9, 148.0, 146.2, 145.9, 144.8, 143.6, 140.7, 128.8 (2C), 128.7 (2C), 126.6, 33.8, 32.3; HRMS-DART (*m/z*): $[M+H]^+$ calcd for $C_{15}H_{12}N_2S_3$, 317.0235, found, 317.0294.

4.2.24. 6,8-Bis(methylthio)pyrrolo[1,2-*a*]pyrazine hydrochloride (2a)

To a solution of **1a** (106 mg, 0.5 mmol) in *t*-BuOH (500 ml) was added *t*-BuOK (0.7 g, 10 mmol) under N_2 atmosphere at 45 °C. After 90 min, the solution was methylated with an excess CH_3I (7.1 g, 50 mmol). The solution was neutralised with dry-ice and evaporated to dryness *in vacuo* at 60 °C. The residue was poured into water (50 ml) and extracted with EtOAc (50 ml). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness giving a brown oil. Column chromatographic purification (*n*-hexane / EtOAc = 15 : 1) yielded **2a** as free base from. The purified compound was added to ~1.25 M HCl methanol solution and stirred at rt for 1 h. The solvent was removed and the residue was precipitated in Et_2O to afford **2a** (12 mg, 10%) as a yellow HCl salt. mp 165 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 8.97 (s, 1H), 8.14 (dd, 1H, J = 4.9, 1.5 Hz), 7.71 (d, 1H, J = 4.9 Hz), 7.09 (s, 1H), 2.42 (s, 3H), 2.32 (s, 3H); HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_9H_{11}ClN_2S_2$, 211.0358, found, 211.0369.

4.2.25. 7-Methyl-6,8-bis(methylthio)pyrrolo[1,2-*a*]pyrazine hydrochloride (2b)

A mixture of **1b** (100 mg, 0.4 mmol) and NaSMe (309 mg, 4.4 mmol) was dissolved in MeOH (10 ml) and 0.01 M aqueous solution of NH_4OAc (10 ml). The mixture was stirred at rt for 3 h. CH_3I (627 mg, 4.4 mmol) was added to the reaction mixture and stirred at rt for 3 h. After completing the reaction (TLC monitoring), MeOH was removed *in vacuo*. The mixture was extracted with EtOAc twice, and the extract was washed with brine, dried over anhydrous Na_2SO_4 and then filtered. The filtrate was concentrated and purified by chromatography on silica gel to yield **2b** as free base form. The purified compound was added ~1.25 M HCl methanol solution and stirred at rt for 1 h. The solvent was removed and the residue was precipitated in Et_2O to afford **2b** (57 mg, 53%) as a yellow HCl salt. mp 162 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 8.99 (s, 1H), 8.21 (dd, 1H, J = 4.9, 1.5 Hz), 7.70 (d, 1H, J = 4.9 Hz), 2.50 (s, 3H), 2.30 (s, 3H), 2.22 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 143.2, 136.0, 131.3, 128.5, 116.3, 115.9, 108.1, 20.5, 17.8, 10.6; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{10}H_{13}ClN_2S_2$, 225.0515, found, 225.0492.

4.2.26. 7-Ethyl-6,8-bis(methylthio)pyrrolo[1,2-*a*]pyrazine hydrochloride (2c)

The compound **2c** was prepared from compound **1c** using the procedure described for **2b**. Yield 77%, yellow solid. mp 157 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 8.99 (d, 1H, J = 1.5 Hz), 8.21 (dd, 1H, J = 4.9, 1.5 Hz), 7.71 (d, 1H, J = 4.9 Hz), 2.96 (q, 2H, J = 7.5 Hz), 2.31 (s, 3H), 2.23 (s, 3H), 1.28 (t, 3H, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 143.5, 142.4, 131.8, 128.9, 116.5, 115.5, 107.7, 21.7, 19.2, 18.5, 16.5; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{11}H_{15}ClN_2S_2$, 239.0662, found, 239.0638.

4.2.27. 6,8-Bis(methylthio)-7-propyl-pyrrolo[1,2-*a*]pyrazine hydrochloride (2d)

The compound **2d** was prepared from compound **1d** using the procedure described for **2b**. Yield 65%, yellow solid. mp 155 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 8.99 (d, 1H, J = 1.5 Hz), 8.21 (dd, 1H, J = 4.9, 1.5 Hz), 7.72 (d, 1H, J = 4.9 Hz), 2.90 (t, 2H, J = 7.8 Hz), 2.30 (s, 3H), 2.22 (s, 3H), 1.75–1.61 (m, 2H), 1.02 (t, 3H, J = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 143.4, 140.7, 131.7, 128.8, 116.5, 115.8, 108.1, 27.9, 25.0, 21.6, 18.3, 14.5; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{12}H_{17}ClN_2S_2$, 253.0828, found, 253.0792.

4.2.28. 7-Butyl-6,8-bis(methylthio)pyrrolo[1,2-*a*]pyrazine hydrochloride (2e)

The compound **2e** was prepared from compound **1e** using the procedure described for **2b**. Yield 85%, yellow solid. mp 153 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 8.99 (d, 1H, J = 1.5 Hz), 8.20 (dd, 1H, J = 4.9, 1.5 Hz), 7.71 (d, 1H, J = 4.9 Hz), 2.92 (t, 2H, J = 7.8 Hz), 2.30 (s, 3H), 2.22 (s, 3H), 1.69–1.60 (m, 2H), 1.44–1.37 (m, 2H), 0.98 (t, 3H, J = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 143.4, 141.0, 131.8, 128.8, 116.5, 115.7, 108.0, 34.0, 25.6, 23.1, 21.6, 18.3, 14.2; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{13}H_{19}ClN_2S_2$, 267.0984, found, 267.0974.

4.2.29. 6,8-Bis(methylthio)-7-phenyl-pyrrolo[1,2-*a*]pyrazine hydrochloride (2f)

The compound **2f** was prepared from compound **1f** using the procedure described for **2b**. Yield 59%, yellow solid. mp 188 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 9.09 (s, 1H), 8.28 (d, 1H, J = 4.9 Hz), 7.77 (d, 1H, J = 4.9 Hz), 7.61–7.40 (m, 5H), 2.13 (s, 3H), 2.11 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 144.5, 139.1, 133.3, 131.5, 130.7 (2C), 129.3, 128.3 (2C), 128.0, 116.7, 116.3, 108.4, 20.9, 18.4; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{15}H_{15}ClN_2S_2$, 287.0671, found, 287.0657.

4.2.30. 7-Benzyl-6,8-bis(methylthio)pyrrolo[1,2-*a*]pyrazine hydrochloride (2g)

The compound **2g** was prepared from compound **1g** using the procedure described for **2b**. Yield 27%, yellow solid. mp 171 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 9.02 (d, 1H, J = 1.5 Hz), 8.22 (dd, 1H, J = 4.9, 1.5 Hz), 7.73 (d, 1H, J = 4.9 Hz), 7.29–7.12 (m, 5H), 4.33 (s, 2H), 2.12 (s, 3H), 2.01 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 143.5, 140.8, 139.2, 131.7, 128.9, 128.7 (2C), 128.3 (2C), 126.0, 116.4, 116.1, 108.3, 31.3, 21.0, 17.7; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{16}H_{17}ClN_2S_2$, 301.0828, found, 301.0857.

4.2.31. 7-(4-Methylbenzyl)-6,8-bis(methylthio)pyrrolo[1,2-*a*]pyrazine hydrochloride (2h)

The compound **2h** was prepared from compound **1h** using the procedure described for **2b**. Yield 92%, yellow solid. mp 190 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 9.01 (d, 1H, J = 1.5 Hz), 8.21 (dd, 1H, J = 4.9, 1.5 Hz), 7.73 (d, 1H, J = 4.9 Hz), 7.15 (m, 2H), 7.05 (m, 2H), 4.29 (s, 2H), 2.28 (s, 3H), 2.13 (s, 3H), 2.03 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 143.7, 139.6, 137.9, 135.6, 131.9, 129.3 (2C), 129.1, 128.8 (2C), 116.6, 116.3, 108.5, 31.1, 21.4, 21.3, 18.0; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{17}H_{19}ClN_2S_2$, 315.0984, found, 315.0967.

4.2.32. 7-(4-Methoxybenzyl)-6,8-bis(methylthio)pyrrolo[1,2-*a*]pyrazine hydrochloride (2i)

The compound **2i** was prepared from compound **1i** using the procedure described for **2b**. Yield 77%, yellow solid. mp 181 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 9.02 (s, 1H), 8.22 (d, 1H, J = 4.4 Hz), 7.73 (d, 1H, J = 4.4 Hz), 7.21–7.16 (m, 2H), 6.82–6.76 (m, 2H), 4.27 (s, 2H), 3.75 (s, 3H),

2.13 (s, 3H), 2.03 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 157.8, 143.3, 139.6, 132.8, 131.6, 129.6 (2C), 128.6, 116.4, 116.1, 113.7 (2C), 108.3, 55.2, 30.4, 21.1, 17.7; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{17}H_{19}ClN_2OS_2$, 331.0933, found, 331.0949.

4.2.33. 7-(4-Chlorobenzyl)-6,8-bis(methylthio)pyrrolo[1,2-*a*]pyrazine hydrochloride (2j)

The compound **2j** was prepared from compound **1j** using the procedure described for **2b**. Yield 35%, yellow solid. mp 182 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 9.02 (d, 1H, J = 1.5 Hz), 8.22 (dd, 1H, J = 4.9, 1.5 Hz), 7.75 (d, 1H, J = 4.9 Hz), 7.24–7.17 (m, 4H), 4.29 (s, 2H), 2.14 (s, 3H), 2.03 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 143.5, 139.3, 138.5, 131.8, 130.0 (2C), 129.0, 128.8, 128.4 (2C), 116.4, 116.0, 108.3, 30.7, 21.1, 17.7; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{16}H_{16}Cl_2N_2S_2$, 335.0438, found, 335.0430.

4.2.34. 6,8-Bis(methylthio)-7-phenethyl-pyrrolo[1,2-*a*]pyrazine hydrochloride (2k)

The compound **2k** was prepared from compound **1k** using the procedure described for **2b**. Yield 55%, yellow solid. mp 173 °C (dec.); ^{1}H NMR ($CDCl_3$, 400 MHz, determined as free base) δ 9.01 (d, 1H, J = 1.5 Hz), 8.19 (dd, 1H, J = 4.9, 1.5 Hz), 7.73 (d, 1H, J = 4.9 Hz), 7.32–7.17 (m, 5H), 3.24 (t, 2H, J = 7.6 Hz), 2.99 (t, 2H, J = 7.6 Hz), 2.28 (s, 3H), 2.11 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 143.5, 142.0, 139.7, 131.8, 128.9, 128.8 (2C), 128.6 (2C), 126.3, 116.6, 116.0, 108.2, 37.8, 28.1, 21.5, 18.1; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{17}H_{19}ClN_2S_2$, 315.0984, found, 315.0987.

4.2.35. 6,7,8-Tris(methylthio)pyrrolo[1,2-*a*]pyrazine hydrochloride (2l)

The compound **2l** was prepared from compound **1a** using the procedure described for **2b**. Yield 51%, yellow solid. mp 161 °C (dec.); ^{1}H NMR (CD_3OD, 400 MHz, determined as free base) δ 9.22 (s, 1H), 8.76 (dd, 1H, J = 5.4, 1.0 Hz), 7.75 (d, 1H, J = 5.4 Hz), 2.70 (s, 3H), 2.57 (s, 3H), 2.54 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz, determined as free base) δ 143.3, 134.4, 128.5, 124.8, 121.3, 116.9, 114.0, 20.8, 19.6, 18.6; HRMS-DART (*m/z*): $[M-Cl]^+$ calcd for $C_{10}H_{13}ClN_2S_3$, 257.0235, found, 257.0229.

4.3. Biological activity screening

4.3.1. Cells and cell culture conditions

Human colon cancer cell line HCT116, were supplied from American Type Culture Collection. The cells were maintained in growth medium containing 10% fetal bovine serum, Dulbecco's modified Eagle's medium and 5% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. For all experiments, HCT116 cells were grown to 80%–90% confluency and were deprived of serum for 16 h before treatment.

4.3.2. Immunoblot analysis

Cell lysates were prepared according to previously published methods.[31] Briefly, cells were lysed in buffer containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.1), 0.1 M NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% Nonidet P-40, 0.5% Triton X-100, 10% glycerol, 1 mM compound **1a**–**k**, or **2a**–**l**, and 0.5 mM phenylmethylsulfonyl fluoride, which was supplemented with a protease inhibitor cocktail (Merck Millipore). Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses were performed as described previously.[32] Immunoreactive protein was visualized using the enhanced chemiluminescence ECL detection kit (Amersham Biosciences). β-Actin served as an internal control to verify equal loading of proteins. Band intensity of the immunoblots was scanned with the Image Scan and Analysis System (Alpha Innotech Co.). The total area of each lane was integrated, and band intensity was determined using Alpha Ease version 5.5 software, followed by background subtraction.

4.3.3. Real-time RT-PCR assay

Total RNA was isolated from cells using Trizol (Invitrogen), and cDNA was synthesized by reverse transcription using an oligo (dT) primer. Then, RT-PCR was conducted with a Light Cycler 1.5 (Roche) using a Light Cycler DNA master SYBR green-I kit, following the manufacturer's instructions. PCR was conducted using primers specifically directed against the genes encoding VEGF (sense, 5′-CCAAGAGTTTGTCTTTCAAC-3′; antisense, 5′-AGAGGCGTTGACATAGGCTT-3′),[33] GLUT-1 (sense, 5′-CGGGCCAAGAGTGTGCTAAA-3′; antisense, 5′-TGACGATACCGGAGCCAATG-3′),[34] HIF-1α (sense, 5′-GTCGGACAGCCTCACCAAACAGAGC-3′; antisense, 5′-GTTAACTTGATCCAAAGCTCTGAG-3′),[35] and β-actin (sense, 5′-CTCTTCCAGCCTTCCTTCCTG-3′; antisense, 5′-CAGCACTGTGTTGGCGTACAG-3′).

4.3.4. Immunoprecipitation assay

To determine HIF-1α ubiquitylation, cells were transfected with the plasmid of His-Ubi. The lysates were incubated with anti-His antibody overnight at 4 °C. After immunoprecipitation, the samples were subjected to SDS-PAGE and were immunoblotted with anti-ubiquitin antibody. The antigen-antibody complex was precipitated following incubation for 2 h at 4 °C with protein G-agarose. The immune complex was solubilized in Laemmli 2 x concentrate buffer and heated for 5 min. The samples were immunoblotted with anti-ubiquitin antibody.

4.3.5. Purification and quantification of miRNAs

Total RNA was prepared as described above. The cDNA was synthesized by using the miScript Reverse Transcription kit (QIAGEN) following the manufacturer's instructions. Real-time RT-PCR assay was conducted using the primers selective for miRNAs of interest: hsa-miRNA-199a-5p, 5′-CCCAGTGTTCAGACTACCTGTTC-3′; hsa-miRNA-20a, 5′-TAAAGTGCTTATAGTGCAGGTAG-3′; hsa-miRNA-20b, 5′-CAAAGTGCTCATAGTGCAGGTAG-3′; hsa-miRNA-17, 5′-CAAAGTGCTTACAGTGCAGGTAG-3′; hsa-miRNA-18a, 5′-TAAGGTGCATCTAGTGCAGATAG-3′; hsa-miRNA-19a, 5′-TGTGCAAATCTATGCAAAACTGA-3′; hsa-miRNA-19b-1, 5′-TGTGCAAATCCATGCAAAACTGA-3′; hsa-miRNA-92a-1, 5′-TATTGCACTTGTCCCGGCCTGT-3′; hsa-miRNA-210, 5′-CTGTGCGTGTGACAGCGGCTGA-3′ and hsa-miRNA-519c-3p, 5′-AAAGTGCATCTTTTTAGAGGAT-3′.

4.3.6. Transfection of miRNAs

miRNA duplexes were prepared as described previously.[36] The following sequences were used to synthesize mimics: miRNA-199a-5p, 5′-CCCAGUGUUCAGACUACCUGUUC-3′ (guide) and 5′-ACAGGUAGUCUGAACACUGGGUA-3′ (passenger) and miRNA-20a, 5′-UAAAGUGCUUAUAGUGCAGGUAG-3′ (guide) and 5′-ACCUGCACUAUAAGCACUUUAAG-3′ (passenger) (Bioneer Co.). Cells were transiently transfected with 100 nM of control mimics (Dharmacon CO.), miRNA-199a-5p mimics or miRNA-20a mimics or a mixture of miRNA 199a-5p and 20a mimics (50 nM each) using FuGENE_HD Reagent (Roche) according to the manufacturer's instruction.

References and notes

1. Brown, J. M.; Wilson, W. R. *Nat. Rev. Cancer* **2004**, *4*, 437.
2. Moulder, J. E.; Rockwell, S. *Cancer Metastasis Rev.* **1987**, *5*, 313.
3. Semenza, G. L. *Nature Rev. Cancer* **2003**, *10*, 721.
4. Semenza. G. L. *Cell* **1999**, *98*, 281.
5. Semenza, G. L. *Physiol.* **2004**, *19*, 176.
6. Clapper, M. L. *Pharmacol. Ther.* **1998**, *78*, 17.
7. Majmundar, A. J.; Wong, W. J.; Simon, M. C. *Mol. Cell* **2010**, *40*, 294.
8. Bos, R.; van der Groep, P.; Greijer, A. E.; Shvarts, A.; Meijer, S.; Pinedo, H. M.; Semenza, G. L.; van Diest, P. J.; van der Wall, E. *Cancer* **2003**, *97*, 1573.
9. Talks, K. L.; Turley, H.; Gatter, K. C.; Maxwell, P. H.; Pugh, C. W.; Ratcliffe, P. J.; Harris, A. L. *Am. J. Pathol.* **2000**, *157*, 411.
10. Zhong, H.; De Marzo, A. M.; Laughner, E.; Lim, M.; Hilton, D. A.; Zagzag, D.; Buechler, P.; Isaacs, W. B.; Semenza, G. L.; Simons, J. W. *Cancer Res.* **1999**, *59*, 5830.
11. Aebersold, D. M.; Burri, P.; Beer, K. T.; Laissue, J.; Djonov, V.; Greiner, R. H.; Semenza, G. L. *Cancer Res.* **2001**, *61*, 2911.
12. Birner, P.; Gatterbauer, B.; Oberhuber, G.; Schindl, M.; Rossler, K.; Prodinger, A.; Budka, H.; Hainfellner, J. A. *Cancer* **2001**, *92*, 165.
13. Birner, P.; Schindl, M.; Obermair, A.; Plank, C.; Breitenecker, G.; Oberhuber, G. *Cancer Res.* **2000**, *60*, 4693.
14. Koukourakis, M. I.; Bentzen, S. M.; Giatromanolaki, A.; Wilson, G. D.; Daley, F. M.; Saunders, M. I.; Dische, S.; Sivridis, E.; Harris, A. L. *J. Clin. Oncol.* **2006**, *24*, 727.
15. Giaccia, A.; Siim, B. G.; Johnson, R. S. *Nat. Rev. Drug Disc*. **2003**, *2*, 803.
16. Lee, K.; Lee, J. H.; Boovanahalli, S. K.; Jin, Y.; Lee, M.; Jin, X.; Kim, J. H.; Hong, Y. -S.; Lee, J. J. *J. Med. Chem.* **2007**, *50*, 1675.
17. Yeo, E. J.; Chun, Y. S.; Cho, Y. S.; Kim, J.; Lee, J. C.; Kim, M. S.; Park, J. W. *J. Natl. Cancer Inst.* **2003**, *95*, 516.
18. Belozerov, V. E.; Van Meir, E. G. *Anticancer Drugs.* **2005**, *16*, 901.
19. Powis, G.; Kirkpatrick, L. *Mol. Cancer Ther.* **2004**, *3*, 647.
20. Folkman, J. *Nat. Rev. Drug Discov.* **2007**, *6*, 273.
21. Kensler, T. W.; Helzlsouer, K. J. *J. Cell. Biochem.* **1995**, *59*, 101.
22. Lee, W. H.; Kim, Y. W.; Choi, J. H.; Brooks, S. C.; Lee, M. -O.; Kim, S. G. *Mol. Cancer Ther.* **2009**, *8*, 2791.
23. Bieder, A.; Decouvelaere, B.; Gaillard, C.; Depaire, H.; Heusse, D.; Ledoux, C.; Lemar, M.; Le Roy, J. P.; Raynaud, L.; Snozzi, C. *Arzneimittelforschung* **1983**, *33*, 1289.
24. Kang, S. G.; Lee, W. H.; Lee, Y. H.; Lee, Y. S.; Kim, S. G. *Carcinogenesis* **2012**, *33*, 661.
25. Curphey, T. J. *Tetrahedron Lett*. **2000**, *41*, 9963.
26. Curphey, T. J. *J. Org. Chem.* **2002**, *67*, 6461.
27. Largeron, M.; Martens, T.; Fleury, M. B. *Tetrahedron* **1987**, *43*, 3421.
28. Zelzer, E.; Levy, Y.; Kahana, C.; Shilo, B. Z.; Rubinstein, M.; Cohen, B. *EMBO J*. **1998**, *17*, 5085.
29. Pan,Y.; Mansfield, K. D.; Bertozzi, C. C.; Rudenko, V.; Chan, D. A.; Giaccia, A. J.; Simon, M. C. *Mol. Cell. Biol.* **2007**, *27*, 912.
30. Treins, C.; Giorgetti-Peraldi, S.; Murdaca, J.; Semenza, G. L. *J. Biol. Chem.* **2002**, *277*, 27975.
31. Cho, M. K.; Kim, W. D.; Ki, S. H.; Hwang, J. –I.; Choi, S.; Lee, C. H.; Kim, S. G. *Mol. Cell. Biol.* **2007**, *27*, 6195.
32. Bae, E. J.; Yang, Y. M.; Kim, J. W.; Kim, S. G. *Hepatology* **2007**, *46*, 730.
33. Zhang, Q.; Tang, X.; Lu, Q.; Zhang, Z.; Rao, J.; Le, A. D. *Mol. Cancer. Ther.* **2006**, *5*, 1227.
34. Ito, S.; Nemoto, T.; Satoh, S.; Sekihara, H.; Seyama, Y.; Kubota, S. *Arch. Biochem. Biophys.* **2000**, *373*, 72.
35. Hayashi, M.; Sakata, M.; Takeda, T.; Tahara, M.; Yamamoto, T.; Minekawa, R.; Isobe, A.; Tasaka, K.; Murata, Y. *J. Clin. Endocrinol. Metab.* **2005**, *90*, 1712.
36. Ogata, A.; Furukawa, C.; Sakurai, K.; Iba, H.; Kitade, Y.; Ueno, Y. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 7299.