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Design, synthesis, and evaluation of indeno[2,1-*c*]pyrazolones for use as inhibitors against hypoxia-inducible factor (HIF)-1 transcriptional activity

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ABSTRACT

HIF-1 is regarded as a promising target for the drugs used in cancer chemotherapy, and creating readily accessible templates for the development of synthetic drug candidates that could inhibit HIF-1 transcriptional activity is an important pursuit. In this study, indeno[2,1-*c*]pyrazolones were designed as readily available synthetic inhibitors of HIF-1 transcriptional activity. Nine compounds were synthesized in 4–5 steps from commercially available starting materials. In evaluations of the ability to inhibit the hypoxia-induced transcriptional activity of HIF-1, compound **3c** showed a higher level compared with that of known inhibitor, YC-1. The compound **3c** suppressed HIF-1α protein accumulation without affecting the levels of HIF-1α mRNA.

1. Introduction

Hypoxia-inducible factor (HIF)-1 is a member of the basic helix-loop-helix proteins of the PER-ARNT-single-minded protein family of transcription factors.[1] HIF-1 regulates the expression of genes involved in angiogenesis, cellular energy metabolism, and cell survival during cancer development. HIF-1 forms a heterodimer with its oxygen-sensitive HIF-1α and a constitutively expressed HIF-1β subunits.[2] Under aerobic conditions (normoxia), proline residues in HIF-1α are hydroxylated by prolyl hydroxylase (PHD),[3] then ubiquitinated by von Hippel-Lindau proteins,[4] and degraded by 26S proteasomes. HIF-1α is also regulated by factor inhibiting HIF (FIH), which is a HIF asparagine hydroxylase.[5] Hydroxylation of the transactivation domains of HIF-1α by FIH promotes avoidance of HIF-1α interactions with p300/CBP coactivator that leads to the repression of HIF-mediated transcription activity.[6] Under low oxygen conditions (hypoxia), the ability of PHD is suppressed and HIF-1α is stabilized. The resultant HIF-1α forms a heterodimer with its β subunit and translocates into the nucleus. HIF-1 binds to hypoxia response elements (HREs) and activates several hundred genes involved in angiogenesis (VEGF), glucose transport (GLUT1), glycolytic pathways (LDH-A), ROS signals (iNOS), erythropoiesis (EPO), and other processes. HIF-1 is, therefore, regarded as a promising target for drugs used in cancer chemotherapy[7] and a variety of HIF-1 inhibitors has been reported.[4i,8] We have also reported diphenylureas[9] and phenoxyacetanilides[10] as HIF-1α inhibitors. Nevertheless, development of a readily accessible template for creating synthetic drug candidates that will inhibit HIF-1 transcriptional activity remains an important pursuit.

Fused-ring systems containing heterocycles are frequently found in the frameworks of marketed drugs.[11] We are interested in 6-5-5 ring systems with a branched 6-membered ring. Indenopyrazole[12] and indenopyrazolone[13] have 6-5-5 + 6 ring systems that are known to exert potent inhibitory activity against Chk1 and CDK, respectively. Our group also reported the EGFR and VEFGR-2 inhibitor indenopyrazolone **1**[14] (Fig. 1) and the structurally similar 6-5-5 + 6 ring system compounds GN02707[9,15] and GN44028[15,16] (structures are not shown) as inhibitors against HIF-1 transcriptional activity and tubulin polymerization. However, synthetic difficulties encountered with the 6-5-5 + 6 ring system compounds have slowed their speed of development as viable drug candidates. Therefore, we designed pyrazolofuropyrazine **2** and demonstrated its synthesis, but it did not exert potent biological activity.[17] Herein, we wish to report the design and the 4-step synthesis for indeno[2,1-*c*]pyrazolones **3**. Nine compounds were synthesized, and compound **3c** exerted potent activity against HIF-1 transcriptional activity during biological evaluation.

2. Results and discussion

2.1. Synthesis

The synthetic plan for **3** is shown in Scheme 1. We planned to

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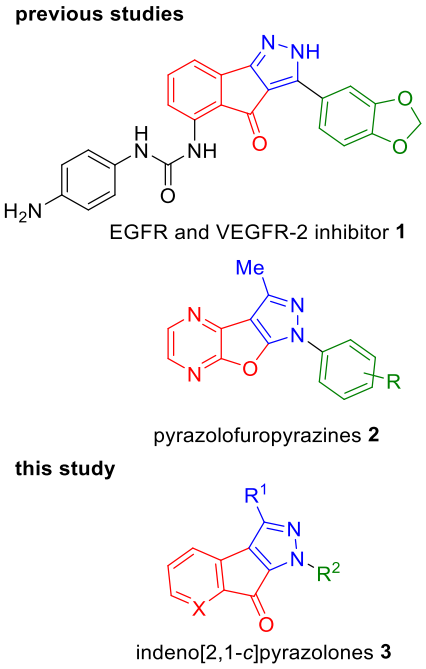
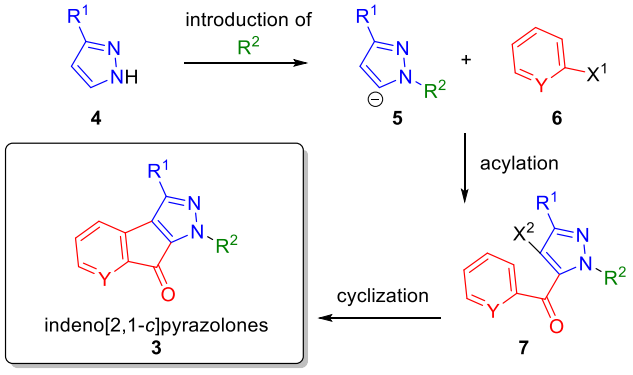


Fig. 1. Chemical structures of 6-5-5 + 6 fused-ring compounds containing heterocycles.

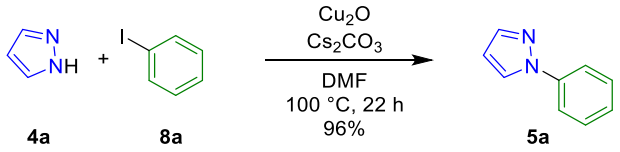
prepare *N*-substituted pyrazole **5** via *N*-alkylation/arylation of **4**. We expected to obtain ketone **7** from nucleophilic addition/nucleophilic acyl substitution of **5** against **6**. Subsequent cyclization was expected to afford the desired indeno[2,1-*c*]pyrazolones **3**.

1-Phenyl-1*H*-pyrazole (**5a**) was prepared via Ullmann-type coupling between **4a** and **8a** in accordance with the reported procedure[18] (Scheme 2).

Two synthetic approaches for pyrazole-containing biaryl ketone **7** have been reported. One of the two approaches used a nucleophilic addition of phenyl Grignard reagent against 5-cyano pyrazole and subsequent hydrolysis of a diaryl imine.[19] Another approach used a nucleophilic acyl substitution of 5-pyrazolyl anion against benzoyl chloride with a pyrazolyl anion prepared from magnesium, zinc chloride, and 5-chloro pyrazole.[20] Unfortunately, synthesis of the substrates used in these known approaches requires multiple steps, which prompted us to examine the direct generation of 5-pyrazolyl lithium from **5a** and its nucleophilic addition/nucleophilic substitution against **6** (Table 1). Alley and Shirley reported that the dropwise addition of 1.02 equiv. of *n*-BuLi at 0 °C in Et_2O led to the lithiation of **5a** at both the 5-position of pyrazole and the *ortho*-position of a phenyl ring.[21] We preliminarily examined lithiation by the dropwise addition of *n*-BuLi into a THF solution of **5a** at −78 °C. The subsequent deuteration using

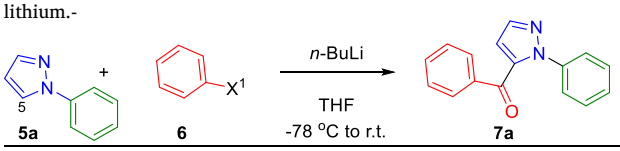


Scheme 1. Synthetic plan for indeno[2,1-*c*]pyrazolones **3**.



Scheme 2. Synthesis of 1-phenyl-1*H*-pyrazole (**5a**) via Ullmann-type coupling.

Table 1
Synthesis of biaryl ketone **7a** using 5-pyrazolyl lithium.-



Entry	6	Yield[a]
1	**6a**: X^1 = COCl	86%
2	**6b**: X^1 = CN	14%
3	**6c**: X^1 = CO_2Bz	15%
4	**6d**: X^1 = CO_2Me	n.r.[b]

[a] Isolated yield.
[b] No reaction.

CD_3OD at −78 °C indicated that the lithiation occurred dominantly at the 5-position of pyrazole. Encouraged by this result, the nucleophilic acyl substitutions of 5-pyrazolyl lithium against electrophiles **6a-6d** were examined. The reaction of 5-pyrazolyl lithium with benzoyl chloride **6a** afforded the desired ketone **7a** in a good yield (entry 1), whereas the reaction with benzonitrile **6b** (entry 2), with benzoic anhydride **6c** (entry 3), and with benzoic acid methyl ester **6d** (entry 4) did not afford satisfactory results (see Table 2).

Unfortunately, the palladium-catalyzed dehydrogenative cyclization conditions reported for the synthesis of fluorenone from benzophenone[22] did not accomplish the synthesis of **3a** from **7a** (Scheme 3). Therefore, **7a** was converted to the corresponding iodopyrazole **9a** using ICl (Scheme 3).[23] Cyclization via direct C−H arylation of **9a** was examined. Copper (I)-catalyzed conditions that were reported for the synthesis of fluorenone[24] only afforded the undesired **7a**. On the other hand, palladium (II)-catalyzed conditions reported for the synthesis of substituted fluorenones[25] afforded the desired **3a** in a 58% yield (2 steps from **7a**).

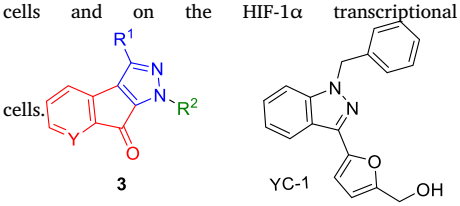
The chemical structure of **3a** was confirmed by ^1H, ^{13}C NMR, IR, and HRMS analysis. In addition, single crystal of **3e** (synthesis is shown later) was obtained and its structure was unambiguously confirmed by X-ray analyses (Fig. 2).[26]

We synthesized indeno[2,1-*c*]pyrazolones **3b-3i** via our developed procedure (Scheme 4). Pyrazole (**4a**) or 3-methyl pyrazole (**4b**) was coupled with aryl halides **8a-8d** to afford *N*-aryl pyrazoles **5a-5d**. The subsequent nucleophilic acyl substitution of pyrazolyl lithium generated by **5a-5d** and commercially available **5e** and **5f** against **6a** afforded the desired ketones **7b-7 g**. The following iodination and intramolecular direct C−H arylation of the ketones **7b-7 g** afforded the desired *N*-substituted indeno[2,1-*c*]pyrazolones **3b-3 g**. In the synthesis of **3e**, an intermediate **7e** was not isolated. Removal of either the methyl group or the methoxy phenyl group from **3d** was performed to afford **3 h** and **3i**.[27]

2.2. Biological evaluation

The synthesized indeno[2,1-*c*]pyrazolones **3a-3i** were tested via a dual luciferase assay for their ability to inhibit HIF-1 transcriptional activity in HeLa cells under hypoxic conditions, and an MTT assay was used to gauge their antiproliferative activity toward HeLa cells under normoxic conditions (Table 1, entries 1–9). YC-1[28] was used as the positive control (entry 10). Under normoxic conditions, HIF-1α is

Table 2
Effects of indeno[2,1-*c*]pyrazolones **3b-3i** on the proliferative activity of HeLa cells and on the HIF-1α transcriptional activity in HeLa cells.

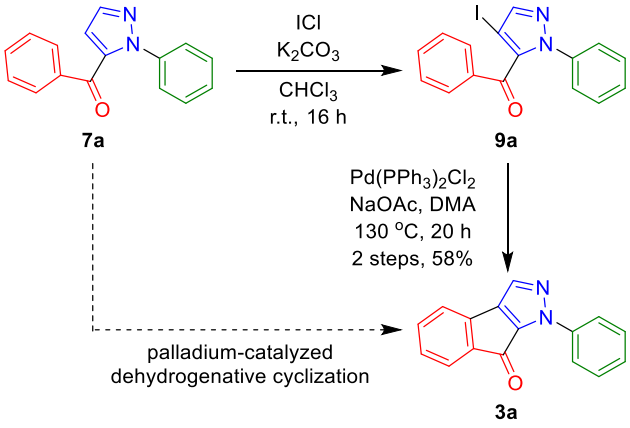


Entry	Compound 3	Y	R^1	R^2	Cytotoxicity[a] IC_{50} (μM)	HRE-Luc[b] IC_{50} (μM)
1	**3a**	C	H	Ph	> 10[c]	1.4 ± 0.83
2	**3b**	N	H	Ph	52 ± 5.7	10 ± 0.46
3	**3c**	C	H	4-MePh	> 10[c]	0.79 ± 0.16
4	**3d**	C	H	4-MeOPh	> 10[c]	0.61 ± 0.10
5	**3e**	C	H	4-CF_3Ph	> 100	23 ± 0.92
6	**3f**	C	Me	Ph	> 30[c]	4.8 ± 0.86
7	**3g**	C	H	Me	49 ± 4.3	8.8 ± 0.079
8	**3h**	C	H	4-HOPh	2.2 ± 0.067	0.39 ± 0.088
9	**3i**	C	H	H	> 30[c]	> 30[c]
10	YC-1				56 ± 0.93	2.4 ± 0.51

[a] HeLa cells were incubated at 37 °C for 72 h in medium containing various concentrations of compounds under normoxic conditions, and cell viability was determined via MTT assay.

[b] HeLa cells transiently transfected with HRE-firefly luciferase and cytomegalovirus promoter-*Renilla* luciferase were incubated at 37 °C for 12 h with compounds under hypoxic conditions. After the supernatant was removed, a luciferase assay was performed using the dual luciferase assay system.

[c] Solubility of these compounds was insufficient, and, their maximum concentrations were set at 10 or 30 μM.



Scheme 3. Synthesis of 1-phenylindeno[2,1-*c*]pyrazol-8(1*H*)-one (**3a**) via intramolecular C–H direct arylation.

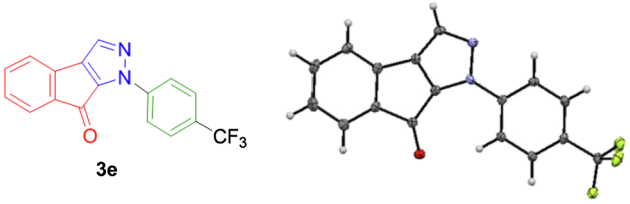


Fig. 2. ORTEP drawing of **3e**.

continuously degraded, and their concentration was expected to be low. Therefore, the compounds that exert low cytotoxicity under normoxic conditions (MTT assay) and high inhibitory activity under hypoxic conditions (reporter gene assay) are candidates for use as HIF-1α inhibitors. The assay results revealed that the compound **3h** exerted potent toxicity under normoxic conditions (entry 8). On the other hand, none of the other compounds exerted potent toxicity. Compounds **3c**, **3d**, and **3h** contained electron-donating groups (-Me, -OMe, −OH) at the *para*-position of the *N*-aryl ring, and exerted potent HIF-1α inhibitory activity (entries 3, 4, and 8). With the exception of **3i** (entry 9), the other compounds, **3a**, **3b**, **3e**, **3f**, and **3g** (entries 1, 2, 5, 6, and 7), exerted medium levels of inhibitory activity. Although the compound **3h** showed the highest level of HIF-1α inhibitory activity (entry 8), it also displayed cytotoxicity under normoxic conditions. These results indicated that compound **3h** inhibited not only HIF-1α transcriptional activity but other biological processes, as well. On the other hand, compounds **3c** and **3d** showed no obvious cytotoxicity under normoxic conditions (entries 3 and 4). A comparison with the reporter gene assay results between compounds **3a** and **3i** (entry 1 vs. 9) clearly indicated the importance of the substituent R^2 for inhibiting HIF-1α transcriptional activity. Introduction of a nitrogen atom as a Y (entry 1 vs. 2), and an electron-withdrawing CF_3 group on the *N*-aryl ring (entry 1 vs. 5) all resulted in detrimental effects against inhibitory activity.

If a compound directly inhibits the luciferase activity, the observed inhibitory activity is not related to HIF-1 transcriptional activity. To verify that possibility, compounds **3c** and **3d** were mixed with luciferase and their luciferase inhibitory activity was investigated. A decrease in the intensity of an emission indicates the inhibitory effect of a compound against luciferase. Compound **3d** had a somewhat strong level of inhibitory activity against luciferase, but no inhibitory activity was observed in the case of compound **3c** (Fig. 3). Thus, the observed inhibitory activity of **3c** against HIF-1α transcriptional activity, as shown in the reporter gene assay, was reliable. We selected compound **3c** as a hit compound.

In order to further elucidate the mechanism of action of compound **3c**, the effects of **3c** on the hypoxia-induced HIF-1α protein accumulation were evaluated by Western blot analysis and the expression of HIF-1α mRNA was evaluated by RT-PCR analysis in HeLa cells (Fig. 4). The compound **3c** suppressed HIF-1α protein accumulation at concentrations higher than 10 μM (Fig. 4a). However, the levels of HIF-1α mRNA were not affected by compound **3c** (Fig. 4b).

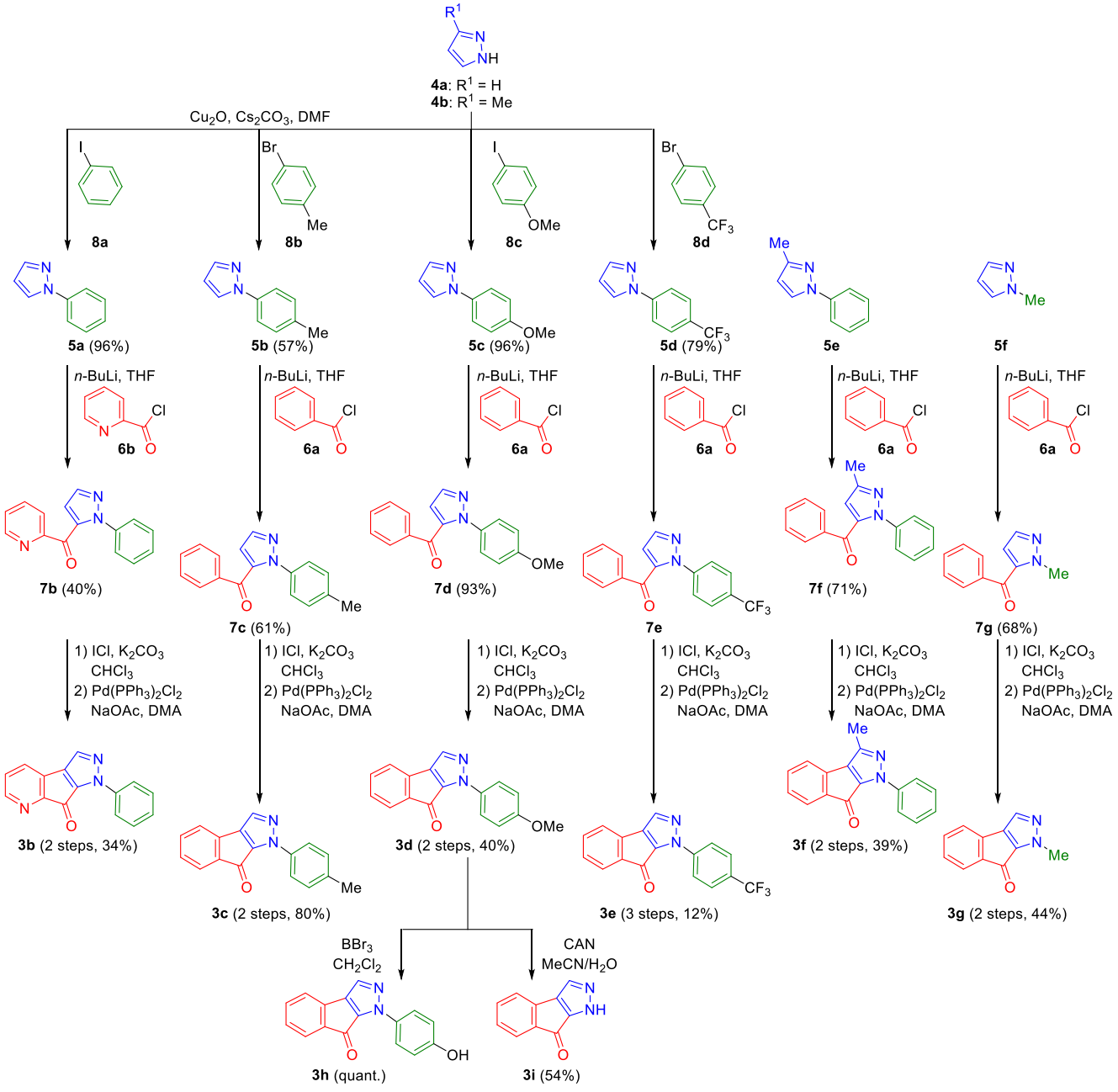
2.3. Conclusions

In summary, we designed indeno[2,1-*c*]pyrazolones to develop readily available synthetic inhibitors against HIF-1 transcriptional activity. Nine compounds were synthesized in 4–5 steps from commercially available starting materials. Evaluation of the ability to inhibit the hypoxia-induced transcriptional activity of HIF-1 revealed that the compound **3c** had a higher level of inhibitory activity compared with that of YC-1. The compound **3c** suppressed HIF-1α protein accumulation without affecting the levels of HIF-1α mRNA. The obtained results showed the usefulness of indeno[2,1-*c*]pyrazolones as a readily available scaffold for future synthetic drug development targeting HIF-1 transcriptional activity or degradation inducing activity.

3. Experimental section

3.1. General methods

NMR spectra were recorded on either using a BRUKER BIOSPIN AVANCE II 400 (400 MHz for ^{1}H, 100 MHz for ^{13}C) or using a BRUKER BIOSPIN AVANCE III HD500 (500 MHz for ^{1}H, 125 MHz for ^{13}C) in the indicated solvent. Chemical shifts are reported in units of parts per million (ppm) relative to the signal (0.00 ppm) for internal tetramethylsilane for solutions in $CDCl_3$ (7.26 ppm for ^{1}H, 77.2 ppm for ^{13}C) or methanol-d_4 (3.31 ppm for ^{1}H, 49.0 ppm for ^{13}C). Multiplicities are reported by using the following abbreviations: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet; and, *J*, coupling constants in Herts (Hz). The IR spectra were recorded on a JASCO FT/IR-4100 with KBr pellets. Only the strongest and/or structurally important peaks are reported as IR data given in cm^{-1}. HRMS (EI-MS) were measured using a JEOL JMS-700. All reactions were monitored by thin layer



Scheme 4. Synthesis of indeno[2,1-*c*]pyrazolones **3b**-**3i**.

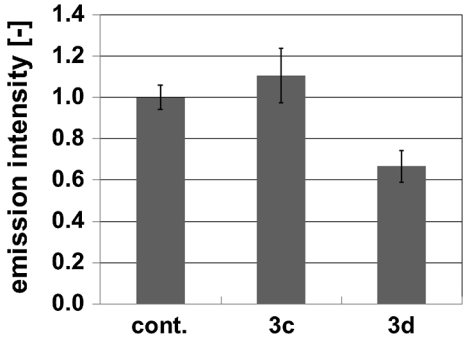


Fig. 3. Inhibitory activity of compounds **3c** and **3d** against luciferase. Luciferase was incubated at 37 °C for 10 min in separate batches of medium that contained one of each of the compounds (1 μM), luciferase assay reagent was added to each mixture, and the emission intensity was measured.

chromatography carried out on 0.2 mm E. Merck silica gel plates (60 F_{254}) with UV light, as visualized by *p*-anisaldehyde solution, 10% ethanolic phosphomolybdic acid, and dinitrophenylhydrazine EtOH solution. Flash column chromatography was performed on silica gel (Fuji Silysia, CHROMATOREX PSQ 60B, 50–200 μm).

3.2. Cell growth assay (MTT assay)

Human cervical carcinoma HeLa cells were used for the cell viability assay. These cells (5 × 103 cells per well of a 96-well plate) were incubated at 37 °C for 72 h in RPMI-1640 medium (100 μL) containing at various concentrations of indenopyrazolones **3a**-**3i** (10 mM DMSO solution). After the incubation, the medium was removed, RPMI-1640 medium (100 μL) and 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS (5 mg/mL, 10 μL) were added to each well, and the cells were further incubated at 37 °C for 2 h. After removal of the medium, DMSO (100 μL) was added and the absorbance at

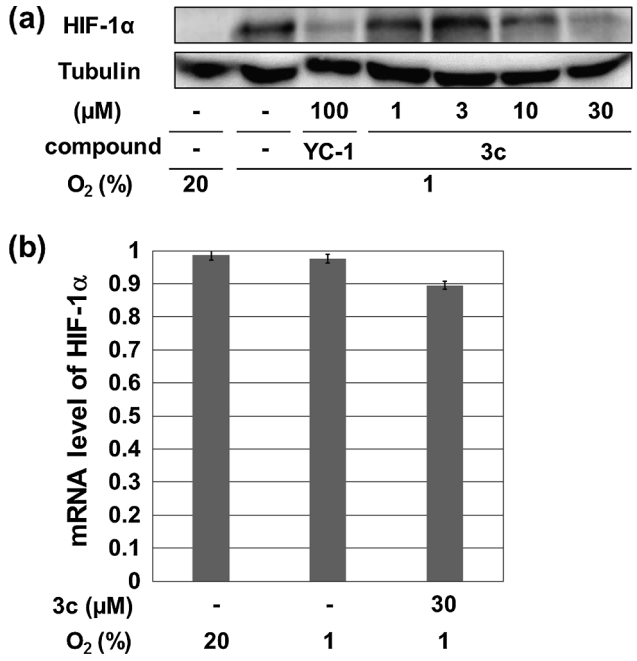


Fig. 4. Effects of compound **3c** on HIF-1α protein and mRNA expression under hypoxic conditions. (a) HIF-1α protein expression was detected by immunoblot analysis with the specific antibody. YC-1 was used as a positive control for the inhibition of HIF-1α protein expression. (b) mRNA level of HIF-1α was detected by RT-PCR.

595 nm was measured with a microplate reader. The drug concentration required to reduce cell viability by 50% (IC_{50}) was determined from semilogarithmic dose–response plots.

3.3. HIF-1 transcriptional activity assay (Luciferase reporter gene assay)

HeLa cells transiently transfected with HRE-Luc[29] and a cytomegalovirus promoter-*Renilla* luciferase (Promega, Madison, WI, USA) reporter genes (2×10^4 cells per well of a 96-well plate) using Lipofectamine 2000 (Thermo Fisher Science, MA, USA) were incubated at 37 °C for 12 h with or without drugs under normoxic or hypoxic (1% O_2) conditions. After removal of supernatant, luciferase assay was performed using a Dual Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The drug concentration required to inhibit relative light units by 50% (IC_{50}) was determined from the semilogarithmic dose-response plots, and the results are means ± SD of triplicate samples.

3.4. Luciferase inhibitory activity assay

To a solution of compound in DMSO and PBS buffer (10 μL), a solution of QuantiLum Recombinant Luciferase (Promega, WI) in PBS (20 ng/μL, 10 μL) was added. After incubation at 37 °C for 10 min, 10 μL of LAR (Promega) was added to the mixture and measured the emission intensity by luminometer infinite F200 (TECAN, Switzerland).

3.5. Western blotting

After drug treatment for 12 h, the cells were dipped in 100 μL of sample buffer (50 mM Tris, pH 7.4, 4% SDS, 10% glycerol, 4% 2-thioethanol, and 50 μg/ml bromophenol blue) for 5 min and the lysate was boiled for 5 min. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare Buckinghamshire, UK), and immunoblotted with anti-HIF-1α antibody (BD Transduction Laboratories, Lexington, KY) and anti-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After further incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, protein expression was visualized with a Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA).

3.6. Quantitative RT-PCR

HeLa cells (1×10^6 cells per well of a 6-well plate) were incubated for 12 h with or without drugs under either normoxic or hypoxic conditions. Total RNA was extracted from the cells using a RNeasy Mini Kit (QIAGEN, German) according to the manufacturer's instructions. After extraction, the extracted RNA (1.0 μg) was reverse transcribed at 40 °C for 50 min by adding 3 μg/μL random R.P. (0.50 μL), 10 mM dNTPs (5.0 μL), 5 × buffer (2.5 μL) and M-MLV (0.50 μL) (Promega, WI). Quantitative RT-PCR was carried out with SYBR Thunderbird (Toyobo, Japan) using the following primers: HIF-1α forward 5′-TTT TCA AGC AGT AGG AAT TGG AA-3′, HIF-1α reverse 5′-GTG ATG TAG TAG CTG CAT GAT CG-3′, β2-microglobulin (B2M) forward 5′-TAC ATG TCT CGA TCC CAC TT-3′, B2M reverse 5′-TAC ACT GAA TTC ACC CCC AC-3′. The mRNA level of B2M was used to normalize the value of HIF-1α.

Acknowledgements

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Declaration of Competing Interest

The authors declare no competing interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2019.115207.

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