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1-[4-(*N*-Benzylamino)phenyl]-3-phenylurea derivatives as a new class of hypoxia-inducible factor-1α inhibitors

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ABSTRACT

A series of 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivatives **2a**–**r** were synthesized as HIF-1α inhibitors. Among the compounds synthesized, compound **2k** was found to be a potent inhibitor against HIF-1α accumulation under hypoxic condition and inhibited the hypoxia-induced HIF-1 transcriptional activity in HEK293 cells (IC_{50} = 7.2 μM). Furthermore, compound **2k** suppressed the hypoxia-induced secretion of VEGF in HeLa cells (IC_{50} = 15 μM).

Angiogenesis is a necessary process for tumors to grow beyond a certain size.[1] The growth of tumoral blood vessels supplies oxygen and nutrients to small tumors, and promotes metastasis. Angiogenesis factors, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), are key growth factors in tumor angiogenesis,[2] therefore, various approaches have been studied for inhibition of these signal transduction to prevent angiogenesis and suppress tumor growth.[3] The major physiological stimulus for VEGF expression is hypoxia, which induces transcription of the VEGF gene by hypoxia-inducible factor (HIF)-1. HIF-1 is known as a heterodimeric complex consisting of a hypoxically inducible subunit HIF-1α and a constitutively expressed subunit HIF-1β. HIF-1β is also known as the arylhydrocarbon nuclear translocator (ARNT), which was originally identified as a binding partner of the aryl hydrocarbon receptor.[4] Under normoxic conditions, HIF-1α protein is subject to oxygen-dependent prolyl hydroxylation, which leads to rapidly degradation by von Hippel–Lindau tumor suppressor protein (pVHL)-mediated ubiquitin-proteasome system (UPS).[5] Under hypoxic conditions, oxygen becomes limiting for prolyl hydroxylase (PHD) activity and HIF-1α is not degradated by UPS. As a result, stabilized HIF-1α binds HIF-1β to form a heterodimeric complex, which binds to the hypoxia response element (HRE) DNA sequence with co-activators to activate various genes including VEGF and EPO.[6] HIF-1α is found at increased levels in a wide variety of human primary tumors compared with corresponding normal tissue,[7a–f] therefore, HIF-1 has been considered as an important target for development of anticancer agents.[8] As shown in Figure 1, various HIF-1 inhibitors that block HIF-1 activation under hypoxic condition has been reported, such as YC-1,[9] topotecan,[10] PX-478,[11] and compound **1**.[12] YC-1 and topotecan are currently undergoing phase I clinical trials on patients expressing high levels of HIF-1α in their tumors.[13]

We have screened a chemical library in our laboratory for compounds that can inhibit HIF-1α accumulation in HeLa cells under hypoxic condition by Western blotting analysis and found that the 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivative **2b** has a potential for block of HIF-1α accumulation. With further modification based on the structure of compound **2b**, we finally discovered compound **2k** as a new class of the HIF-1α inhibitor.

As shown in Scheme 1, 4-nitroaniline was treated with Boc_2O, and the protected aniline **4** was converted the diamine **5** by hydrogenation. Urea formation of **5** with phenylisocyanate, which was prepared from aniline and triphosgene in situ, followed by deprotection of the Boc group with trifluoroacetic acid afforded 1-(4-aminophenyl)-3-phenylurea **7**.[14]

Reductive amination of **7** with various aldehydes **8** was carried out by using $NaCNBH_3$ in MeOH to give 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivatives **2** in 14–99% yields (Scheme 2 and Table 1). The aldehydes **8j**, **8q** and **8r** were prepared from **8j**, 3-formyl-4-hydroxybenzoic acid and trimesic acid, respectively.[15,16] Although esterification of **2h** and **2i** in methanol gave the corresponding methyl esters **2k** and **2l**, respectively, **2g** underwent cyclization under this condition to give **9** in 99% yield.

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The synthesized 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivatives **3a**–**r** were tested for inhibitory effect on HIF-1α accumulation in HeLa and HCT116 cells at a 30 μM concentration of compounds under hypoxic conditions (1% O_2, 94% N_2, and 5% CO_2) by Western blot analysis. YC-1[7] was used as a positive control for comparison. Among compounds **2a**–**f** which have either hydroxy or methoxy groups on the benzyl moiety of molecules, compounds **2d** and **2e** exhibited relatively potent HIF-1α inhibition and the inhibitory potency of them was almost the same as that observed by YC-1 in HeLa and HCT116 cells under hypoxic condition (Fig. 2a and b). Among the carboxylic acids and their methyl esters (**2g**–**l**), compound **2k** showed the most potent HIF-1α inhibition under hypoxic condition; the corresponding *ortho*- and *para*-isomers (**2j** and **2l**) and carboxylic acids **2g**–**i** were less potent than compound **2k** (Fig. 2c and d). Disubstituted derivatives **2m**–**r** were also tested for inhibitory effect on HIF-1α accumulation under hypoxic condition. Compound **2o**, which has 2,3-dimethoxy groups on the benzyl moiety, showed a similar inhibition to compounds **2d** and **2k** in HeLa cells, although the other disubstituted compounds showed less potent (Fig. 2e and f). From these results, compound **2k** possesses the highest HIF-1α inhibition among the compounds synthesized, and the inhibitory potency is higher than that of YC-1 [**2k**: density ratio (HIF-1αβ) = 0.39 (HeLa) and 0.39 (HCT116) versus YC-1: density ratio (HIF-1αβ) = 0.47 (HeLa) and 0.52 (HCT116)].

To confirm the inhibition of transcriptional activity under hypoxic condition, we have investigated the effect of synthesized compounds on hypoxia-induced transcriptional activation of HIF-1 using cell-based reporter assay in recombinant Human Embryonic Kidney (HEK) 293 cells. YC-1 was used as a positive compound for a comparison and the IC_{50} value was 9.24 μM in HEK293 cells. Compounds **2a**–**h**, **2j**, **2m**, **2n**, and **2p**–**r** showed moderate inhibition of hypoxia-induced transcriptional activation by HIF-1 (15–70 μM), and *para*-carboxylic acid **2i** and its methyl ester **2l** did not exhibit inhibitory activity at a 100 μM concentration of compounds. Compounds **2k** and **2o**, which showed potent HIF-1α inhibition in HeLa cells under hypoxic condition, also exhibited significant inhibition of hypoxia-induced transcriptional activation by HIF-1, and their IC_{50} values were 7.21 and 8.62 μM, respectively (Table 1).

We next examined concentration-dependent inhibition against hypoxia-induced HIF-1α accumulation by western blot analysis and mRNA expression level of HIF-1α by RT-PCR analysis in HeLa cells.[10] As shown in Figure 3a, compound **2k** suppressed HIF-1α accumulation in a concentration-dependent manner without affecting the expression level of HIF-1β protein. However, **2k** did not suppress the level of HIF-1α mRNA (Fig. 3b). The effect of compound **2k** on hypoxia-induced VEGF secretion was also measured using ELISA. As shown in Figure 4, compound **2k** significantly inhibited the hypoxia-induced secretion of VEGF at a concentration-dependent manner, and the IC_{50} value was calculated as 15 μM.

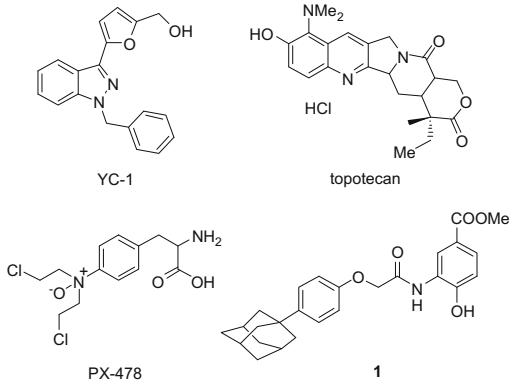
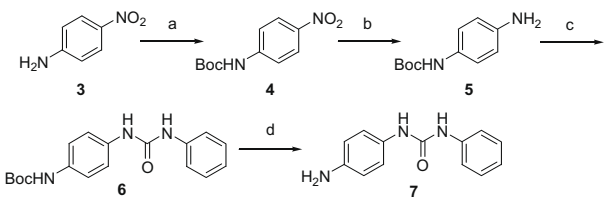
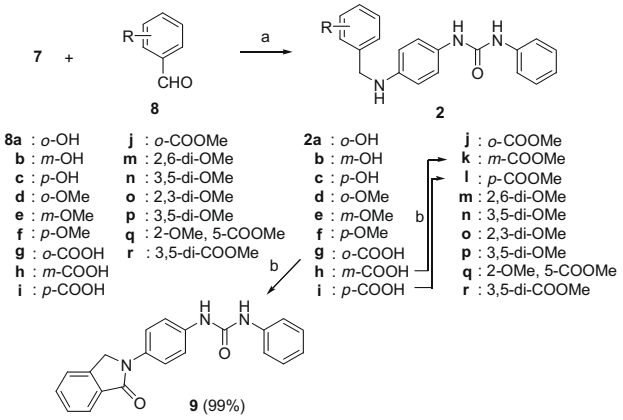


Figure 1. Structures of HIF-1 inhibitors.

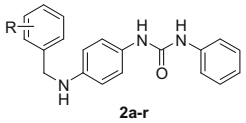


Scheme 1. Reagents and conditions: (a) Boc_2O, CH_2Cl_2, reflux, 99%; (b) H_2, Pd/C, MeOH, 58%; (c) phenylisocyanate, toluene, reflux, 99%; (d) TFA, CH_2Cl_2, reflux, 91%.



Scheme 2. Reagents and conditions: (a) $NaCNBH_3$, MeOH, reflux; (b) MeOH, cat. H_2SO_4, reflux.

Table 1
Chemical yields and in vitro inhibition of HIF-1 transcriptional activity in cell-based HRE reporter assay



R	Compd	Yield (%)	IC_{50}/μM [a]
o-OH	**2a**	53	32.7 ± 5.72
m-OH	**2b**	14	69.9 ± 11.9
p-OH	**2c**	14	35.5 ± 8.06
o-OMe	**2d**	40	17.4 ± 4.74
m-OMe	**2e**	36	52.1 ± 3.29
p-OMe	**2f**	51	23.0 ± 9.03
o-COOH	**2g**	25	51.7 ± 1.96
m-COOH	**2h**	57	41.6 ± 3.22
p-COOH	**2i**	32	>100
o-COOMe	**2j**	46	32.5 ± 20.9
m-COOMe	**2k**	53[b]	7.21 ± 0.67
p-COOMe	**2l**	97[b]	>100
2, 6-OMe	**2m**	30	39.1 ± 5.72
3,5-OMe	**2n**	38	18.7 ± 0.57
2,3-OMe	**2o**	99	8.62 ± 2.06
2,5-OMe	**2p**	99	29.6 ± 1.85
2-OMe, 5-COOMe	**2q**	45	22.1 ± 7.52
3,5-COOMe	**3r**	78	15.1 ± 2.32
	YC-1		9.21 ± 3.24

[a] The cells were incubated for 16 h with or without drugs under normoxic or hypoxic condition. After removal of supernatant, the luciferase assay was performed using Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. The drug concentration required to inhibit RLU (Relative Light Unit) by 50% (IC_{50}) was determined from semilogarithmic concentration–response plots and results represent the mean ± SD of triplicate samples.

[b] Yields of esterification from **2h** or **2i**.

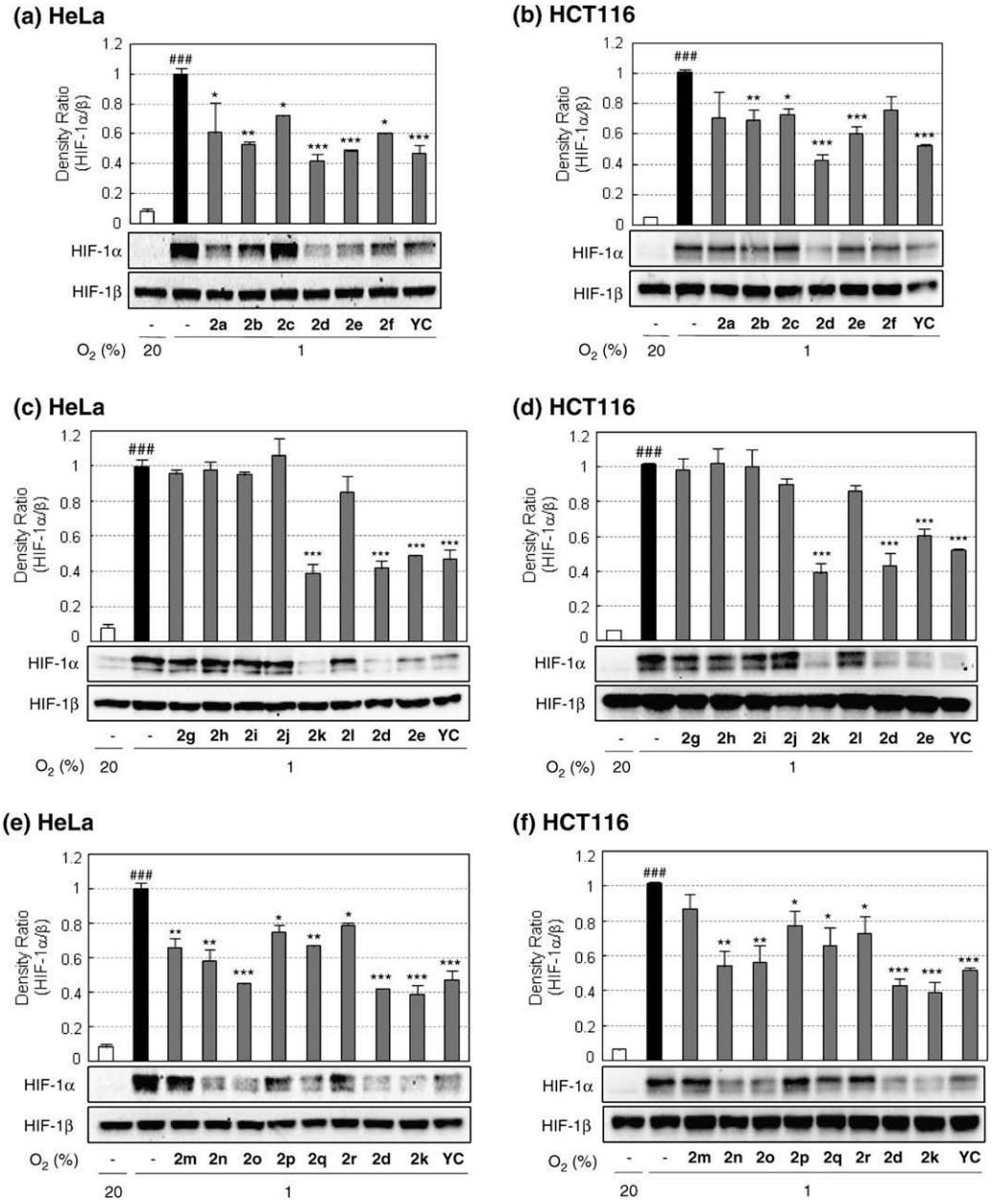


Figure 2. Relative ratios of HIF-1α and -1β levels by Western blot analyses at 30 μM concentration of compounds. The levels of each protein were detected by immunoblot analysis with the HIF-1α or HIF-1β specific antibodies after 4 h-incubation of cells with compounds. The density ratios of HIF-1α protein level to HIF-1β were calculated, and the mean value of density ratio in hypoxia control group was set to 1.0. Values are the means from triplicate experiments with the SEM shown by vertical bars. Statistical significance: ###: $P < 0.001$ versus normoxia control; *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$ versus hypoxia control.

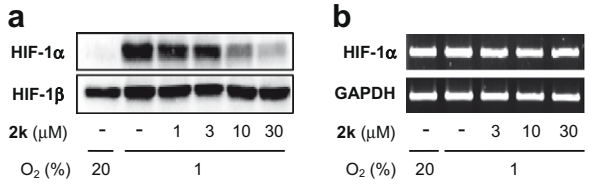


Figure 3. Effect of **2k** on the accumulation of the HIF-1α protein and mRNA induced by hypoxia. (a) HeLa cells were incubated with compound **2k** at different concentrations under hypoxic condition for 4 h. HIF-1β was used as the loading control and '–' is DMSO as control. The levels of each protein were detected by immunoblot analysis with the HIF-1α or HIF-1β specific antibodies. (b) RT-PCR analysis for the effect of compound **2k** on the hypoxia-induced mRNA level of HIF-1α in HeLa cells for 2 h. GAPDH was used as the loading control and '–' is DMSO as control.

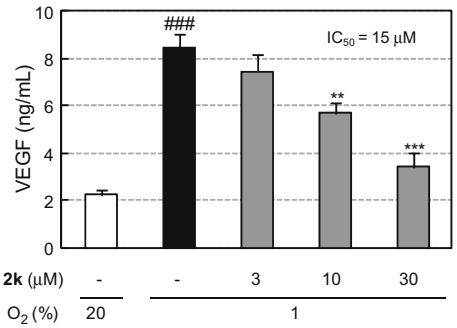


Figure 4. Compound **2k** selectively inhibited hypoxia-induced secreted VEGF protein in HeLa cells. The cells were incubated for 12 h with or without drugs. Values are the means from triplicate experiments with the SEM shown by vertical bars. Statistical significance: ###: $P < 0.001$ versus normoxia control; *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$ versus hypoxia control.

In conclusion, we have found that compound **2k** is the most potent inhibitor of the hypoxia-induced HIF-1α protein accumulation. Furthermore, we have confirmed that compound **2k** showed the significant suppression of the hypoxia-induced transcriptional activity (IC_{50} = 7.21 μM) and VEGF secretion (IC_{50} = 15 μM). These results suggest that 1-[4-(*N*-benzylamino)phenyl]-3-phenylurea derivative **2k** will be a new class of HIF-1α inhibitor. Further detailed mechanistic studies of HIF-1α inhibition by **2k** is currently under investigation in our group.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.122.

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