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Synthesis and Biological Evaluation of 2-Aminoisonicotinic Acid Analogues as HIF-1α Inhibitors

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The hypoxia inducible factor 1 (HIF-1) is a heterodimeric transcription factor which is an important regulator of growing tumor's response to hypoxia. It was reported that HIF-1 activates genes that allow the cancer cells to survive and grow in the hostile hypoxic tumor environment. Furthermore, the activity of HIF-1 has been shown to be central to regulation of apoptosis and cell survival, cell adhesion and extracellular matrix turnover, cytoskeletal structure, cell motility, epithelial homeostasis, vascular tonus, eythropoiesis and iron metabolism, metabolic homeostasis, and pH regulation.

HIF-1 is a heterodimeric protein that consists of a constitutively expressing nuclear-located β-subunit, also termed aryl hydrocarbon nuclear translocator (ARNT) and a O_2-dependently regulated α-subunit.[1,2] Intratumoral hypoxia and genetic alterations can lead to HIF-1α over-expression.[3] In animal models, HIF-1α overexpression is associated with increased tumor growth, vascularization, and metastasis, whereas HIF-1α loss-of-function has the opposite effect; these findings validate HIF-1α as an attractive cancer drug target.[3,4] Recently, considerable effort has been directed to the discovery of HIF-1 inhibitors, from chemical libraries and natural products.[5-7] These inhibitors reportedly regulate the HIF-1 signaling pathway through a variety of molecular mechanisms, including transcriptional regulation, folding, stabilization, nuclear translocation, degradation, and transactivation.

Our studies have been centred on development of small molecule HIF-1α inhibitors, including compounds **1-3**, as potential anticancer agents (Fig. 1).[8-11] Of interest, these compounds were found from a structure-activity relationship study using a cell based HRE-Luc assay. Particularly, the 2-aminoisonicotinamide analogue **2** displayed potent inhibition of hypoxia-induced accumulation of HIF-1α protein in human cancer cell lines, in addition to a dose-dependent inhibition of HIF-1 target genes, such as *VEGF*. Herein, we present our focused structure-activity relationship study of HIF-1α inhibitors based on the isonicotinic moiety using human colorectal carcinoma cells HCT116.

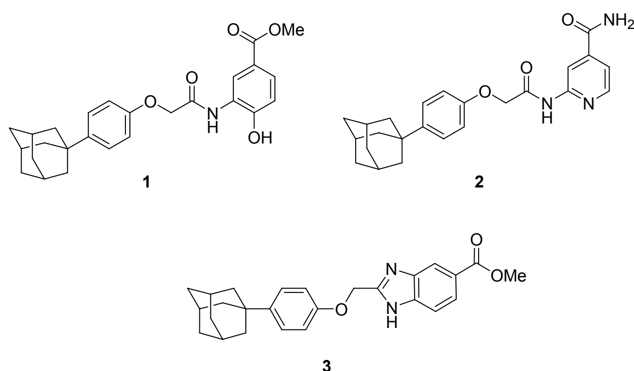
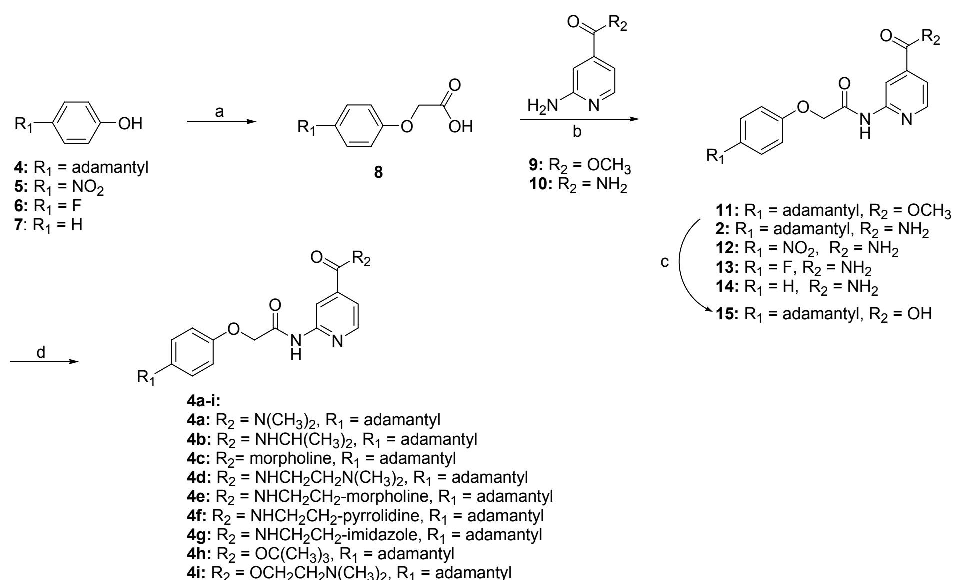


Figure 1. Small molecule HIF-1α inhibitors.

[a]These authors equally contributed to this work.

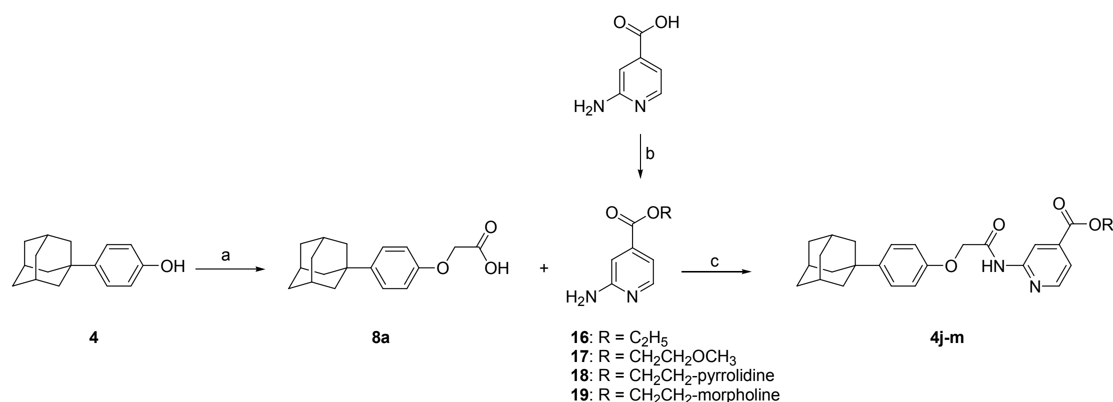
The general routes for the synthesis of 2-aminoisonicotinic acid analogues are outlined in Schemes 1 and 2. As shown in Scheme 1, reaction of a variety of phenols **4-7** with ethyl chloroacetate followed by alkaline hydrolysis afforded the corresponding phenoxy acetic acid **8** in quantitative yield. Among the phenoxy acetic acid compounds, 4-admantanyl phenoxy acetic acid **8a** was reacted with commercially available 2-amino isonicotinic methyl ester **9** and 2-aminoisonicotinamide **10** to yield compounds **11** and **2**, respectively. Under same condition other phenoxy acetic acids **8b-d** were coupled with 2-amino-isonicotinamide **10** to give the corresponding amide derivatives **12-14**. Reaction of an ester analogue **11** in the presence of lithium iodide in pyridine produced the corresponding phenoxy acetic acid analogue **15**, which was coupled with amines to give the corresponding amide derivatives **4a-f** under PPAA and Et_3N condition in good yields. Esterification of **15** with *tert*-butanol by using DCC and DMAP afforded compound **4h**. As for preparation of **4i**, intermediate **15** reacted with alkyl halide utilizing K_2CO_3 and Cs_2CO_3 to give compound **4i**. Scheme 2 described synthetic procedure of ester derivatives possessing 2-amino-isonicotinate moiety. For synthesis of intermediates **16-19**, commercially available 2-aminoisonicotinic acid was reacted with alkyl halides under basic condition. Analogues **4j-m** were also readily obtained from 4-adamantyl phenoxy acetic acid **8a** and the corresponding intermediates **16-19** in the presence of PPAA and Et_3N.

The *in vitro* HIF inhibitory activities of 2-aminoisonicotinic acid analogues **2**, **4a-m** and **11-14** were evaluated using cell based HRE-Luc assay at two concentrations (1 μM and 10 μM), as shown in Table 1.[12] Topotecan, a known small-molecule HIF-1α inhibitor, was used as a reference compound for a comparision, which showed 56.9% of HRE-Luc activity at 3 μM. In this study, human colon cancer cells HCT116 was chosen because of its dramatic change in accumulation of HIF-1α protein



Reagents and conditions: (a) i. Ethyl chloroacetate, K_2CO_3, DMF; ii. LiOH, THF, H_2O; (b) PyBOP, DMAP, DMF (c) LiI, pyridine; (d) alkylamines, PPAA, Et_3N, CH_3CN for **4a-g**; DCC, DMAP, *t*-BuOH for **4h**; alkyl halide, K_2CO_3, Cs_2CO_3 for **4i**

Scheme 1



Reagents and conditions: (a) i. Ethyl chloroacetate, K_2CO_3, DMF; ii. LiOH, THF, H_2O; (b) alkyl halides, $NaHCO_3$, acetone; (c) PPAA, Et_3N, CH_3CN

Scheme 2

under hypoxia, in comparison with that in normoxic condition. In order to define the key structural requirements for HIF-1α inhibition of 2-aminoisonicotinic acid analogues, our optimization strategy was centered on two discrete areas: (1) the 2-aminoisonicotinic acid, which could be derivatived further and (2) the adamantyl phenyl ring which could be replaced by a phenyl ring with various groups. In view of the moderate inhibitory activity of the amide **2** (HRE-Luc activities; 97% at 1 μM, 43% at 10 μM) in cell based HRE-Luc assay using HCT116 cells, we first explored the effect of altering the amide group by preparing compounds **4a-g**, in which dimethylamino, isopropylamino and morpholyl group attached *via* amide bond. Compound **4d** (88% at 1 μM, 44% at 10 μM) and **4g** (89% at 1 μM, 32% at 10 μM), linked through two carbon and three carbon chain, respectively, demonstrated considerable potency in HCT116, while **4a** showed a marked decrease in inhibitory potency. The isoniconicotinic ester derivative **11** displayed considerable potent inhibition of HIF-1α. Encouraged by this, the impact of modification of the isonicotinic acid moiety was further extended to the analogue series characterized by an ester substitution. This derivatization produced ester analogues **4h-m**, comprising ethyl and *tert*-butyl attached *via* ester group, in which **4h** (48% at 1 μM, 41% at 10 μM) showed significant inhibitory activity. However, compounds **4i** and **4k-m**, linked through two carbon chain, exhibited poor inhibitory activity suggesting that the linker space is important for potency. Subsequently, we examined the effects of different aromatic substituents on the adamantyl phenyl ring, retaining the carboxylamide group at the meta position of pyridine ring. However, compounds **12-14** with various functionalities, including nitro, fluoro and proton, displayed poor inhibitory activity.

Based on the initial *in vitro* HIF inhibitory assay, compound **4g** was chosen as a representative compound for further study. Isonicotinamide analogue **4g** showed the inhibitory activity in

Table 1. Structure-activity relationship of 2-aminoisonicotinic acid analogues

no.	structure		HRE-Luc activity (%)	
	R_1	R_2	1 μM	10 μM
2[9]		$CONH_2$	97	43
11[9]		$COOCH_3$	95	49
12	NO_2	$CONH_2$	> 100	> 100
13	F	$CONH_2$	> 100	> 100
14	H	$CONH_2$	> 100	> 100
4a			102	84
4b			95	64
4c			99	80
4d			88	44
4e			97	87
4f			85	81
4g[9]			89	32
4h		$COOC(CH_3)_3$	48	41
4i			100	97
4j		$COOC_2H_5$	93	83
4k			103	99
4l			98	77
4m			104	85
Topotecan			56.9 (3 μM)	

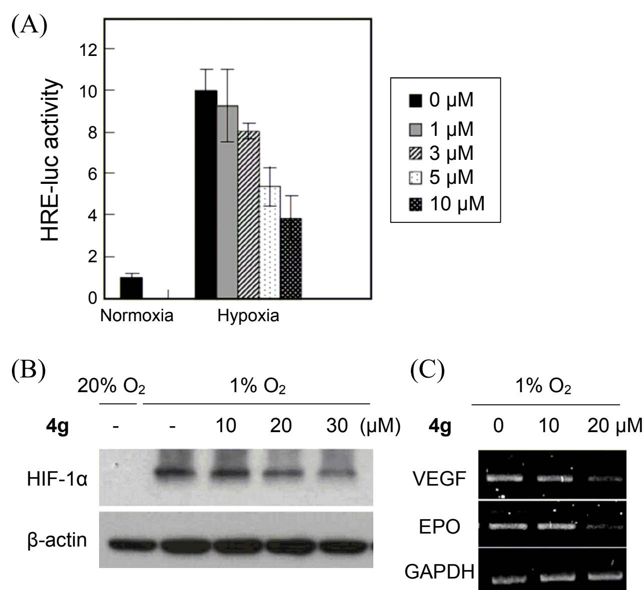


Figure 2. Effect of HIF-1α inhibitor **4g**. (A) Dose dependent inhibition of HRE-Luc activity by **4g**; (B) Western blot analysis for the effect of compound **4g** on the accumulation of HIF-1α protein in HCT116 cells under hypoxia. β-actin was used as load control and 0 μM represents DMSO as a control; **C.** RT-PCR analysis for the effect of compound **4g** on the hypoxia-induced mRNA expression of *VEGF* and *EPO* in HCT116 cells. GAPDH was used as load control and 0 μM represents DMSO as a control.

cell based HRE-Luc assay in a dose-dependent manner as shown in Figure 2A and its IC_{50} was determined to 4.3 μM. In order to confirm its HIF inhibition, compound **4g** was evaluated by Western blot analysis for its effect on hypoxia-induced HIF-1α accumulation. As shown in Figure 2B, the inhibitor **4g** blocked HIF-1α accumulation dose-dependently in human carcinoma cancer cell lines HCT-116. HIF-1α responds to the hypoxia by binding to the HRE of target genes. Accordingly, the inhibitory effect of **4g** on HIF-1α protein was analyzed *via* the expression of the downstream target genes *VEGF* and *EPO*, which are associated with the angiogenesis of an aggressive tumor. In the presence of **4g**, the mRNA levels of *VEGF* and *EPO* in HCT 116 cells were decreased, while the mRNA expression level of the control *GAPDH* was not affected (Fig. 2C). Further study is, however, warranted for more detailed mechanism of action of **4g** on HIF-1α inhibition under hypoxia. Considering that poor pharmacokinetic properties accounted for many drug failures in development, our compound was tested for its drug-like property, i.e. cell permeability using Caco-2 cells, which are most

Table 2. Caco-2 permeability of **4g**

compound	test conc. (μM)	transport direction	$P_{app}{}^{a} \times 10^6$ (cm/sec)		recovery (%)		permeability
			mean	SD	mean	SD	
4g	5	A-to-B	22.9	2.2	75.9	4.0	high
Metoprolol	10	A-to-B	27.2	0.5	ND[c]		high permeability standard
Ranitidine	20	A-to-B	0.6	0.01	ND[c]		low permeability standard

[a]P_{app} values were determined in HBSS supplemented with 10 mM HEPES (pH 7.4). Assays were conducted in triplicate. [b]Samples were analyzed by API2000 LC/MS/MS system in MRM mode. [c]ND: not determined.

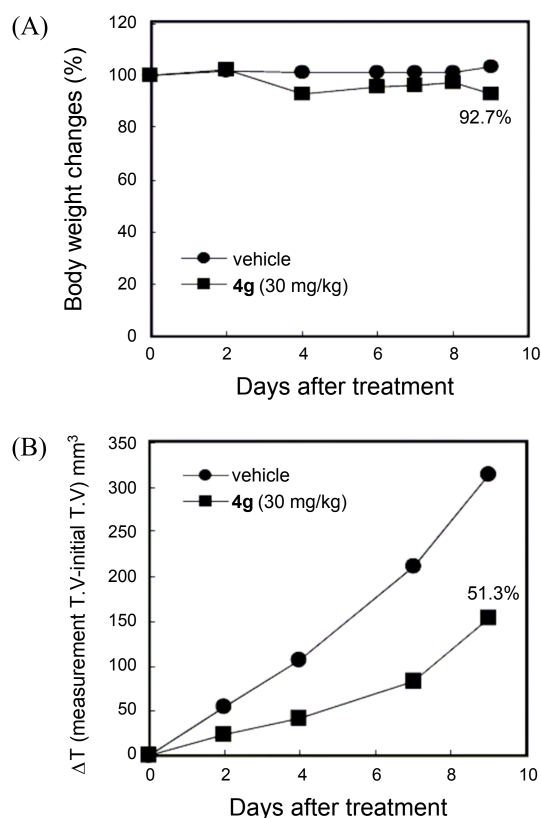


Figure 3. *In vivo* antitumor activity of **4g**. (A) Changes in body weight; (B) Effect of **4g** on tumor growth inhibition.

commonly used in pharmaceutical study to predict cell absorption of drug candidate. As shown in Table 2, **4g** showed high permeability (P_{app} values 22.9 × 10^6 cm/sec at 5 μM) compared with the positive control metoprolol (P_{app} values 27.2 × 10^6 cm/sec at 10 μM) and the negative control ranitidine (P_{app} values 0.6 × 10^6 cm/sec, 20 μM) on Caco-2 cells. The high permeability of **4g** may contribute to utility of this type of compounds for development of preclinical candidates. Encouraged by the biological activity and pharmacokinetic property, *in vivo* antitumor activity of **4g** was further evaluated using nude mice and the human cancer cell line HCT116. The results were outlined in Figure 3. When the mice were treated intravenously with **4g** (30 mg/kg), tumor growth was inhibited by 51.3%, compared to the vehicle treated control group. Compound **4g** did not cause any severe side effect, but the body weight decreased to 92.7%, which might be caused by minor adverse effect.

In summary, a series of 2-aminoisonicotinic acid analogues have been synthesized and their *in vitro* HIF-1 inhibitory activity was evaluated using cell based HRE-Luc assay in human colorectal carcinoma cells HCT116. Especially, compound **4g** exhibited dose-dependent inhibition of HIF-1α, target gene expressions under hypoxia, favorable permeability, and potent *in vivo* efficacy. With these results in hand, further molecular mechanism studies are in progress, which will be reported in due course.

Experimental Section

General procedure for coupling reaction (4a-g and 4j-m). Compound **15** or **8a** (1 equiv) was suspended in acetonitrile and Et_3N (4.0 equiv), 50% PPAA (1.2 equiv) were added at room temperature. The reaction solution was stirred at room temperature for 30 min and the amine (1.2 equiv) was added. The reaction solution was stirred overnight and evaporated under reduced pressure. The residue was purified by column chromatography to obtain the products (**4a-g** and **4j-m**).

Other synthetic procedures and all spectral data of 2-aminoisonicotinic acid analogues can be found in supplementary material. The detailed *in vitro* and *in vivo* assay procedures were also shown in supporting information.

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