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Development of novel inhibitors targeting HIF-1α towards anticancer drug discovery

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ABSTRACT

Hypoxia-inducible factor-1α (HIF-1α) is a critical regulatory protein of cellular response to hypoxia, and regulates the transcription of many genes involved in key aspects of cancer biology, including immortalization, maintenance of stem cell pools, cellular dedifferentiation, vascularization, and invasion/metastasis. HIF-1α has been implicated in the regulation of genes involved in angiogenesis, for example, VEGF and is associated with tumor progression. In the last decade, over expression of HIF-1α has been demonstrated in many common human cancers and emerging as a validated target for anticancer drug discovery. Here we report the discovery of newly designed and synthesized pyridylpyrimidine based potent and selective inhibitors of HIF-1α. P2630 has been found as potent antiproliferative, antiangiogenic and orally efficacious compound in PC-3 xenograft mice model.

Hypoxia-inducible factor-1 (HIF-1) is a heterodimer of α and β transcriptional factor, and a master regulator of transcriptional response to oxygen deficiency. HIF-1 has been implicated in the regulation of genes involved in tumorigenesis, angiogenesis, glycolysis, survival, growth, invasion, metastasis, to treatment resistance.[1–4] The over expression of HIF-1α is associated with advanced disease stage and poor prognosis in many common human cancers.[5] The HIF-1α subunit is degraded rapidly in normoxic conditions and stabilized under hypoxic conditions, while HIF-1β is constitutively expressed as a nuclear protein. Under normoxia HIF-1α protein undergoes hydroxylation at specific prolyl residue by prolylhydroxylase (PHD), this hydroxylated HIF-1α is recognized by von Hippel-Lindau (VHL) tumor suppressor protein and undergoes for proteasomal degradation.[6] Under hypoxia, PHD is not activated and hence the hydroxylation by PHD is compromised, which leads to HIF-1α stabilization. Stabilized HIF-1α translocates to the nucleus and forms a heterodimeric complex with HIF-1β. This complex further binds to hypoxia response element (HRE) DNA sequence with co-activators to activate various genes involved in angiogenesis (through VEGF), erythropoietin (EPO), glycolysis etc.[1–4,7] In addition to hypoxia, the activation of oncogenes and/or inactivation of tumor suppressor genes can also lead to HIF-1 activation.[1–4] In general, the availability and activity of HIF-1α protein determines the bioactivity of HIF-1 protein.[8] HIF-1α is now considered as a potential target for many common solid tumors.[2] Various advance molecules targeting HIF-1α are under clinical trials, for example, Topotecan,[9] PX478,[10] YC-1.[11] Recently boron-containing phenoxyacetanilide based molecule (GN26361),[12] AC1-001[13] and its benzimidazole derivative AC-004[14] have been reported as lead molecules targeting HIF-1α. In the present study, we herein report the design, synthesis, and discovery of pyridylpyrimidine based inhibitors of HIF-1α. In our quest for finding potential therapeutics, we choose pyridylpyrimidine as a basic scaffold. The substituted amino-pyridyl and pyridylpyrimidine scaffold is a very common structural motif that can be found in many natural products and in several pharmacologically interesting compounds.[15–17] Therefore, the synthesis of pyridylpyrimidine derivatives, with the objective of developing new drugs, is an active area of research.[18] Based on the above rationale we designed, synthesized and characterized various pyridylpyrimidine based molecules[18] (**3a–g**, **4a–h**, **5a–e**, **6a–e**, and **7**) as out lined in Scheme 1. In brief 5-aminopicolinonitrile (**1**) was treated with ethanolic-HCl followed by ethanolic-ammonia till basic pH. This reaction mixture was stirred at room temperature for about 12–15 h to get 4-amino-picolinimidamide hydrochloride (**2**). Compound **2** on treatment with substituted β-ketoester in ethanol/water (1:2) mixture and Na_2CO_3 at room temperature for 22 h yielded corresponding 2-(5-aminopyridin-2-yl)pyrimidin-4-ol based compounds (**3a–g**) in fairly good yield (Scheme 1). Compounds **3a–g** were subjected

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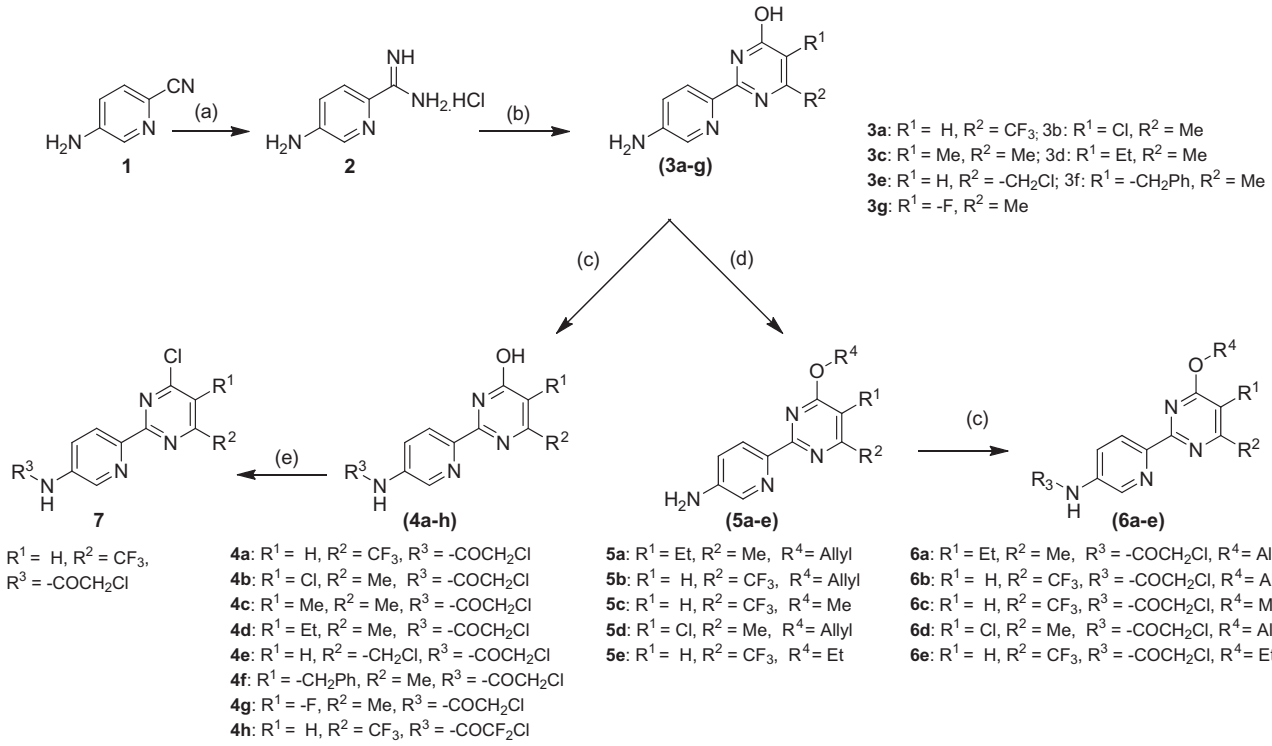

Scheme 1. Reagents and conditions: (a) ethanol, HCl gas till saturation, stir at rt for 30 min, then addition of ethanolic-ammonia at −20 °C till basic pH, then stir at rt for about 12–15 h; (b) substituted β-ketoester, ethanol/water (1:2), Na_2CO_3, rt for 22 h; (c) triethyl amine, substituted acetyl chloride in THF, rt, 1 h; (d) alkyl halide, K_2CO_3, DMF, rt, 1 h; (e) benzyl triethyl ammonium chloride, dimethylaniline, $POCl_3$, CH_3CN, reflux, 3 h.

to acylation of amine using various acetyl chlorides in THF at room temperature for 1 h, yielded compounds **4a**–**h**. Compound **4a** was further refluxed in acetonitrile for 3 h using benzyltriethylammonium chloride and $POCl_3$ to get compound **7**. In another set of reaction **3a**–**g** were treated with various alkyl halides and K_2CO_3 in DMF at room temperature for 1 h to get compounds **5a**–**e**. Compounds **5a**–**e** on further acylation yielded compounds **6a**–**e**. All these newly synthesized compounds were characterized by ^{1}H NMR, MS, HRMS and purity (>99%) by HPLC.

These newly synthesized compounds were screened against HIF-1α by reporter gene based assay under hypoxic (1% O_2) and normoxic (21% O_2) conditions.[9] Compounds **4a**, **4d**, **4e**, **6d** and **7** have shown significant inhibition of HIF-1α under hypoxia (⩽1.5 μM). Further, these HIF-1α active compounds were subjected to reporter gene based assay using U251-pGL3 cell line under normoxia to evaluate their specificity index (SI) as shown in Table 1. The higher SI indicates the specificity of compounds in hypoxia with respect to normoxia. Compound **4a** was found as the most potent and specific HIF-1α inhibitor with 10-fold SI and further referred to as P2630. The inhibition of HIF-1α expression was further confirmed by Western blot analysis using U251-HRE and PC-3 cell lines in a dose dependent manner[19] (Fig. 1).

After confirming the HIF-1α activity, compound **4a** (P2630) was subjected for antiproliferative activity using ^{3}H-thymidine incorporation assay across different cancer cell lines such as prostate cancer (PC-3 and DU-145), glioblastoma (U251), colon cancer (HCT-116), ovarian cancer (Ovcar-3), pancreatic cancer (Panc-1), and normal cell lines like MRC-5 and WI-38 (Table 2).

P2630 has shown the best antiproliferative activity in PC-3 cell line (IC_{50}; 1 μM) whereas the antiproliferative IC_{50} in MRC-5 and WI-38 (normal cells) were >10 and 6.5 μM, respectively. These data suggest that P2630 has selective potential to inhibit HIF-1α expression under hypoxia over normoxia and specifically inhibits the proliferation of cancer cells. Since it is known that HIF-1 regulates the genes involved in angiogenesis, we thought to evaluate its effect on VEGF mRNA expression under hypoxia in PC-3 cells. The inhibition of VEGF mRNA expression by P2630 in dose dependent

Table 1
HIF-1α inhibitory activity of new compounds **3a**–**g**, **4a**–**h**, **5a**–**e**, **6a**–**e**, and **7** under hypoxia and normoxia

Compd No.	HIF-1α[a] (hypoxia)	Normoxia[a]	SI
3a	>30	ND	ND
3b	>30	ND	ND
3c	>30	ND	ND
3d	>30	ND	ND
3e	6	22	3.6
3f	>30	ND	ND
3g	>30	ND	ND
4a (P2630)	0.8	8	10
4b	5.5	ND	ND
4c	>30	ND	ND
4d	1.5	5.5	3.6
4e	1.5	5.5	3.6
4f	4	10	2.5
4g	2	6	3
4h	>30	ND	ND
5a	>30	ND	ND
5b	>30	ND	ND
5c	>30	ND	ND
5d	>30	ND	ND
5e	>30	ND	ND
6a	1.8	22	12.2
6b	2	30	15
6c	3.5	15	4.3
6d	1.5	8	5.3
6e	8	ND	ND
7	1.4	10	7.1
Topotecan	0.06	>3	>50

[a] IC_{50} values in μM; ND: not done; SI: specificity index. Topotecan evaluated as positive control.

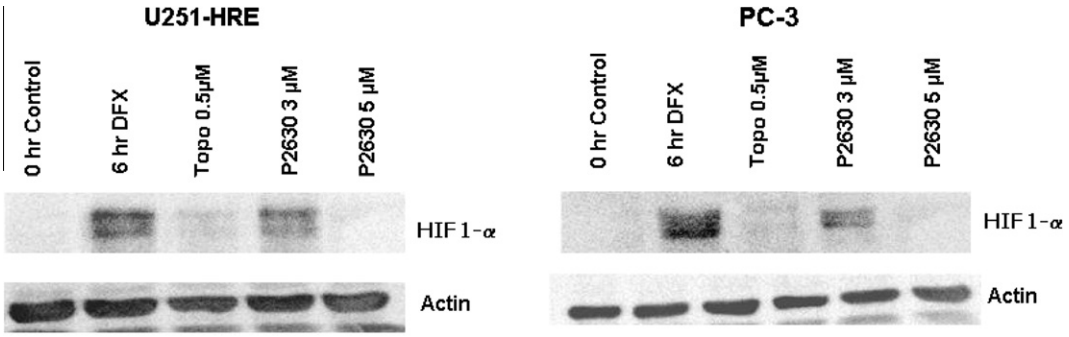

Figure 1. Western blot analysis showing the effect of P2630 on HIF-1α protein expression in U251-HRE and PC-3 cell lines. P2630 specifically inhibited HIF-1α protein accumulation under hypoxia in a dose dependent manner in both the cell lines.

Table 2
Anti-proliferative activity (IC_{50} in μM) of P2630 against human cancer cell lines & normal cell lines (MRC-5 & WI-38)

Compound	PC-3	HCT-116	U251	Ovcar-3	DU-145	Panc-1	MRC-5	WI-38
P2630	1.0	0.8	1.5	3.5	1.2	2.0	>10	6.5
Topotecan	1.0	ND	0.5	ND	1.2	ND	ND	ND

ND: not done.

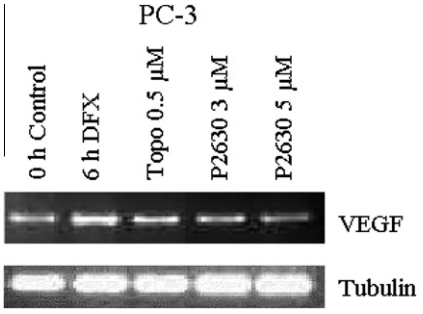

Figure 2. RT-PCR analysis showing the down regulation of VEGF mRNA by P2630 under hypoxia in PC-3 cell line in dose dependent manner at 3 and 5 μM. Topotecan was used as a positive control.

manner was confirmed by RT-PCR as shown in Figure 2. The antiangiogenic potential of P2630 was evaluated by 3D gel endothelial tube formation assay as per published procedure.[20] Topotecan was used as positive control in this study (Fig. 3). Result shows that P2630 significantly inhibits the HUVEC tube formation at 3 and 5 μM in dose dependent manner.

Considering the antiproliferative potential of P2630 in PC-3 cancer cells and its antiangiogenic potential, the in-vivo efficacy was evaluated in PC-3 xenograft model using SCID mice. P2630 administered 50 mg/kg, bid, po for 19 days, which shows significant tumor growth inhibition (TGI) as compared to vehicle treated animals (Fig. 4A). The mice treated with P2630 have no significant weight loss during course of treatment (Fig. 4B).

In conclusion, we report the discovery of P2630,[21] a structurally novel inhibitor of HIF-1α. Compound P2630 displays very good potency in both mechanistic and antiproliferative cellular assays. Compound P2630 also exhibits significant oral in-vivo efficacy in PC-3 xenograft model without any significant weight loss. The further mechanistic studies of this molecule are warranted and will be published in due course of time.

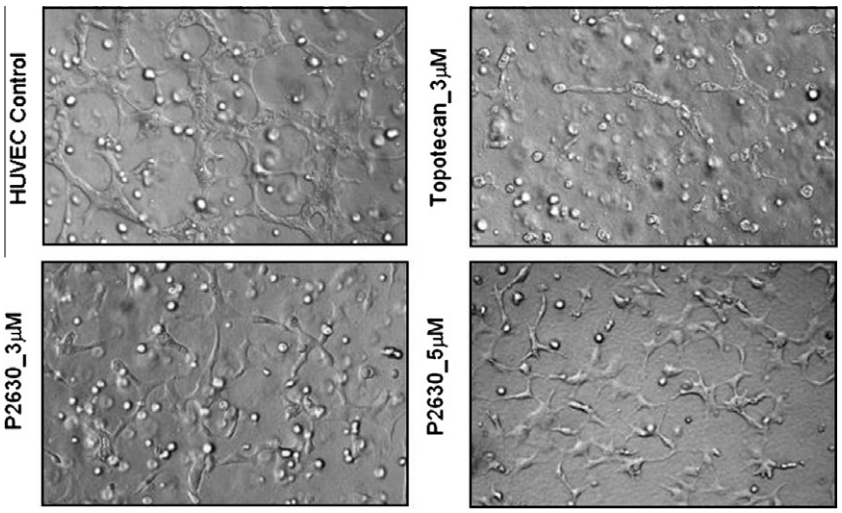

Figure 3. The antiangiogenic potential of P2630 by endothelial tube formation assay. P2630 was demonstrated to have significant inhibitory effect on HUVEC tube formation in a dose dependent manner (3 and 5 μM) Topotecan has been taken as positive control.

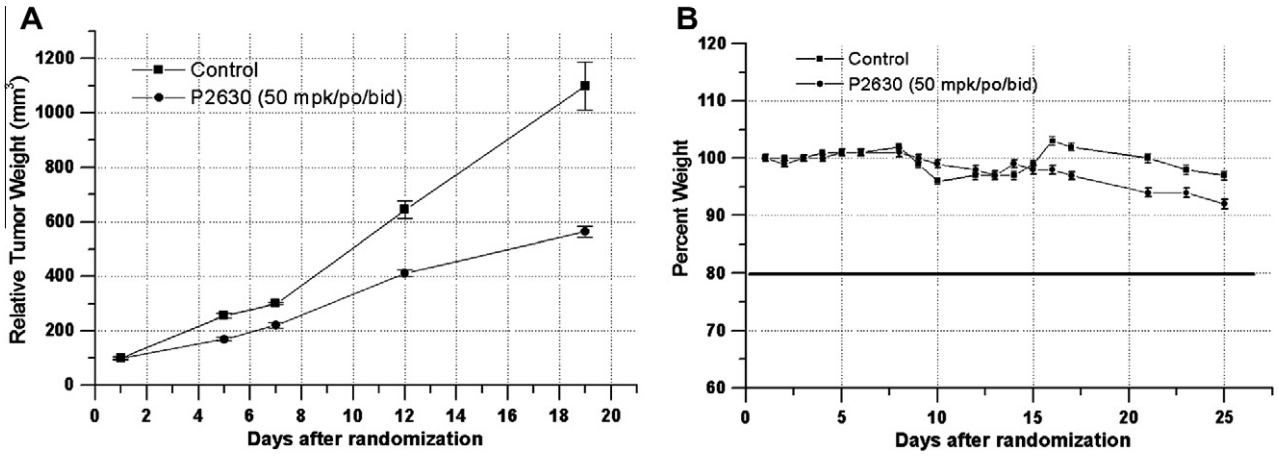

Figure 4. In vivo efficacy of P2630 in PC-3 human tumor xenograft. (A) SCID mice were dosed twice a day for 19 days. Tumor size (median tumor volume) was measured on 5th, 7th, 12th, and 19th day and compared w.r.t tumor volume of vehicle treated mice. (B) Percentage weight profile of P2630 treated and vehicle treated mice during course of treatment.

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21. *Spectral data for P2630* (**4a**): 2-chloro-*N*-(6-(4-hydroxy-6-(trifluoromethyl)-pyrimidin-2-yl)pyridin-3-yl)acetamide: ^{1}H NMR (DMSO-d_6, 300 MHz): δ: 12.80 (br s, 1H), 10.93 (s, 1H), 8.97 (s, 1H), 8.25–8.34 (m, 2H), 6.90 (s, 1H), 4.37 (s, 2H); MS: m/e (ES−) 331.02 (M−1); HRMS calcd for $C_{12}H_8ClF_3N_4O_2$ 332.0288; found: 333.0361 (M+H); HPLC purity 99.88%.