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Discovery of a New Molecular Probe ML228: An Activator of the Hypoxia Inducible Factor (HIF) Pathway

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Abstract

Hypoxia and ischemia are linked to several serious public health problems that affect most major organ systems. Specific examples include diseases of the cardiovascular, pulmonary, renal, neurologic, and musculoskeletal systems. The most significant pathway for cellular response to hypoxia is the hypoxia inducible factor (HIF) pathway. HIFs are transcription factors responsible for the activation of genes which encode proteins that mediate adaptive responses to reduced oxygen availability. A high-throughput cell-based HIF-mediated gene reporter screen was carried out using the NIH's Molecular Libraries Small Molecule Repository to identify activators of the HIF pathway. This communication describes the subsequent medicinal chemistry optimization of a triazine scaffold that led to the identification of the new molecular probe **ML228**. A discussion of HIF activation SAR within this chemotype as well as detailed *in vitro* characterization of the probe molecule is presented here.

Hypoxia and ischemia are linked to several serious public health problems that affect most major organ systems. Specific examples include diseases of the cardiovascular, pulmonary, renal, neurologic, and musculoskeletal systems. The hypoxia inducible factor (HIF) pathway is the major pathway required for intracellular adaptation initiated by lower oxygen availability in the bloodstream. The HIF pathway is known to take part in the angiogenesis processes enhancing blood supply required for tissue repair and regeneration. A unique gene transcription program involving the activation of multiple transcriptional factors known as the hypoxia inducible factors (HIFs) is triggered in response to hypoxia.¹ HIFs exist as a heterodimeric complex containing one of three α subunits (HIF-1 α , HIF-2 α , or HIF-3 α) associated with the aryl hydrocarbon receptor nuclear translocator (ARNT), also known as HIF-1 β . Although the hypoxic response requires multiple HIF subunits to be functional, hypoxia only leads to changes in both the accumulation and the activity of the HIF-1 α subunit.

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In most cells the HIF pathway is primarily regulated by the inhibition of the HIF-1 α subunit degradation during hypoxia.² In normoxia, the intracellular level of HIF-1 α protein is typically low due to its on-going ubiquitination and proteasomal degradation. The degradation of the HIF-1 α subunit is initiated through hydroxylation on a conserved proline residue, which is a process mediated by three prolyl hydroxylase (PHD) isoforms known as PHD1, PHD2, and PHD3.³ The presence of iron, oxygen, and 2-oxoglutarate (2-OG) are required for hydroxylation. Prolyl hydroxylation is blocked during hypoxia, which leads to the stabilization of HIF-1 α and the establishment of a negative feedback loop whereby hypoxia and HIF-1 α both upregulate PHD2 expression.⁴ Once HIF-1 α is stabilized, it accumulates in the cells, dimerizes with ARNT, and translocates into the nucleus. Inside the nucleus, the HIF-1 α /ARNT complex through interaction with its binding partners initiates the transactivation of HIF-responsive genes, such as the glucose transporter Glut1 and the angiogenic factor vascular endothelial growth factor (VEGF).⁵ Several strategies have been explored for manipulation of the HIF pathway at various points to either promote or retard angiogenesis.⁶

To date, the most common strategy for HIF activation is through inhibition of PHDs. General iron chelators such as desferrioxamine (DFO) and the inorganic salt cobalt chloride (CoCl₂) have been explored clinically. DFO activates HIF-1 α by chelating iron, whereas cobalt displaces iron from PHDs. Both compounds have been shown to increase HIF activation *in vitro* and *in vivo* and have demonstrated an ability to promote angiogenesis and produce a similar response to ischemia.⁷ Although these agents have been investigated clinically in certain disease settings, they have also been associated with toxicity.⁸ As such, more recent approaches to PHD inhibition have centered on small molecules.⁹ In many cases, known PHD inhibitors contain functional groups that are isosteric analogs of 2-OG (Figure 1). Common examples are *N*-acylated glycine analogs such as **1** and **2**.¹⁰ Most known PHD inhibitors contain a carboxylic acid group, and in the case of compounds such as pyrazole **3**, this functional group participates in a key interaction that mimics that of 2-OG.¹¹ PHD inhibitors without a carboxylic acid group, such as **4** and **5**, possess other acidic proton donors.¹²

In order to identify novel and interesting HIF activators, a phenotypic cell-based high-throughput screen was conducted on the NIH's Molecular Libraries Small Molecule Repository, which contains more than 300K compounds. The primary screening assay utilized a previously described cell-based gene reporter assay with stably transfected human U2OS osteosarcoma cell line expressing luciferase under control of hypoxia response elements (HREs).¹³ DFO (100 μ M) was used as a positive control, with a previously determined EC₅₀ of 17.8 μ M in the same assay. Compounds were initially screened in the HRE-luciferase assay at a concentration of 7.5 μ M. Using a cutoff at 10% or more of the activity obtained with the positive control DFO identified approximately 1200 primary hits. Complete dose response curves were subsequently generated for these compounds using the primary HRE-luciferase assay. These compounds were also tested in a high content imaging assay examining the accumulation and nuclear translocation of HIF-1 α in USOS cells expressing HIF-1 α fused to the Green Fluorescent Protein (GFP).¹⁴ Finally, each of these compounds was also submitted to a commercially available cell-based proteasome inhibition assay in order to eliminate any compounds acting as general proteasome inhibitors.¹⁵

Among the compounds emerging from the screening process, isoxazoles **6** and **7** and triazines **8** and **9** represented particularly interesting starting points for chemistry as they were dissimilar to known PHD inhibitor scaffolds and lacked the acidic functional groups common to such scaffolds (Figure 2). Such facts increased the potential likelihood that these compounds may activate HIF through alternative mechanisms to PHD inhibition, or if they did act through PHD, may at the very least bind in a much different manner. Furthermore,

recent reports have demonstrated that HIF activation increases cell viability and potentiates dopamine release in dopaminergic cells, which may indicate a potential application in certain neurodegenerative disorders such as Parkinson's disease.¹⁶ Since highly acidic functional groups can often limit the ability of small molecules to penetrate the blood-brain barrier, new chemical starting points for HIF activators without such functionality are considered interesting.

A 35 member amide library was rapidly prepared through standard coupling conditions with commercially available 5-phenylisoxazole-3-carboxylic acid and structurally diverse amines. In addition to the aforementioned HRE-luciferase and high content imaging nuclear translocation assays, the compounds were also tested in a real time PCR assay for their ability to induce VEGF transcription.¹⁴ Compounds were also subjected to the proteasome inhibition assay and were found to be generally inactive. The activity of the isoxazole series in the HRE-luciferase assay was uniformly disappointing. Representative results with some of the active compounds are shown here (Table 1). In fact, resynthesis of hit **6** revealed that compound to be 3–4 fold less potent than originally indicated. Although further work could certainly be done within this chemotype, early results in the triazine series were more compelling and focus was directed there.

The synthetic route for the synthesis of triazine HIF activators such as **8** and **9** was relatively straightforward (Scheme 1).¹⁷ Reaction of 2-cyanopyridine **13** with hydrazine afforded imidohydrazide **14**, which was subsequently reacted with ethyl 2-oxo-2-phenylacetate in a condensation reaction to provide triazin-5(4*H*)-one **15**. Treatment with phosphorous oxychloride under thermal conditions provided the chloride **16**, which was directly reacted with commercially available amines in an S_NAr reaction to afford the final compounds.

An initial library of 24 compounds (including resynthesized batches of **8** and **9**) focused on variation of the amine moiety was prepared within the triazine series, and results there were much more promising (Table 2). As with the isoxazole series, these compounds were also inactive in the proteasome inhibition assay. The potency of the resynthesized batches of **8** and **9** closely matched those found during analysis of the initial hits. Not surprisingly, cyclopropylmethanamine **17** was similar in potency to **8**. Increasing the size of the aliphatic group on the amine (**18** and **19**) led to improvements in potency relative to **8**. Such modifications led to increasingly lipophilic compounds; however, incorporation of the more polar functional groups (**20–22**) was not favorable. Tertiary amine analogs of hit **9** were also prepared, but these analogs (**23–24**) failed to improve potency. Some heteroaryl secondary amine analogs such as **25** and **26** were also prepared but were also not improvements. Although, the simple benzyl amine analog **27** had similar activity to hit **8**, it was noted that methyl substitution of the benzyl group at any of the positions on the aromatic ring (**28–30**) enhanced potency relative to **27**. Having an initial survey of the SAR in hand, attention was turned to the synthesis of a second generation library that would refine the SAR around the amine moiety. First, additional aliphatic amine analogs would be prepared in an attempt to improve upon analogs **18** and **19**. Second, more *meta* and *para* substituted analogs of **27** would be prepared.

Selected aliphatic amine analogs of **18** and **19** are presented here (Table 3). Introduction of a methyl group on the sp³ carbon adjacent to the secondary amine of **18** afforded analogs **31** and **32**. This modification had little effect on activity, and any preference for one enantiomer over the other was minimal. Wanting to continue to explore opportunities to reduce lipophilicity, tetrahydropyran **33** was prepared; however, such a modification again failed to yield positive results. Replacement of the *t*-butyl group of **19** with phenyl gave analog **34**, which was similarly potent in the primary assay but less active in the secondary assays. Modification of **34** by installation of a pyridyl ring (**35**) reduced activity. Clearly, polar

functional groups, whether installed in saturated or aromatic systems, were not advantageous. Additional *meta* and *para* substituted analogs of **27** are also shown here (Table 4). Both 3,4-dimethylphenyl analog **36** and 3-chlorophenyl analog **37** were noted improvements over both **27** and previously prepared methylphenyl analogs **29** and **30**. Conversely, methoxyphenyl analogs **38** and **39** were less active than **36** and **37**. Substitution of **27** with a *para*-*t*-butyl group (**40**) gave one of the most potent compounds tested. As was demonstrated in other analogs, attempts to introduce polarity through installation of pyridine rings (**41** and **42**) significantly reduced activity. Installation of a phenyl ring at either the *meta* (**43**) or *para* (**ML228**) position of the benzyl amine **27**, on the other hand, quite enhanced potency.

ML228 was selected for further characterization since it showed the most improved potency within the chemotype and was viewed as an interesting tool compound. The probe compound **ML228** was among the top active compounds in each of the three HIF-specific activity assays and was inactive in the proteasome inhibition counterscreen assay (Figure 3). In order to gain an understanding of the broad pharmacology of this compound, **ML228** was submitted to a radioligand binding assay panel of 68 GPCRs, ion channels, kinases, and transporters.¹⁸ **ML228** was found to inhibit greater than 75% of radioligand binding in six assays when tested at a concentration of 10 μ M (Table 5). An additional ten molecular targets were identified where binding inhibition was moderate (50–75% inhibition),¹⁹ while there was no significant binding (<50% inhibition) at the remaining 52 targets. Lipophilic compounds such as **ML228** sometimes exhibit non-specific binding in assays such as these; however, this binding assay profile provides some basic information for future users of the probe compound to consider.

Most compounds that are known to activate HIF act through metal chelation. In order to provide some further insight into the possible mechanisms by which **ML228** acts on the HIF pathway, the role of metal chelation was investigated (Figure 4). To achieve this task, excess iron or zinc (50 μ M) were added in the media with the probe and tested in the primary HRE-luciferase assay. Results were compared to those with only media and probe. The presence of iron produced a dramatic effect, shifting the dose response curve to the right and substantially decreasing the magnitude of response. The addition of zinc produced a much smaller rightward shift and the magnitude of response was similar to compound alone. These results suggest that **ML228** can chelate iron, which is consistent with some known mechanisms of HIF activation. A cell toxicity assay²⁰ was also performed in the same cell line and ruled out that the changes in activity observed with the addition of these metals was due to cell death (data not shown).

Recent publication of PHD inhibitor **3** and related analogs disclosed x-ray co-crystal structures of some inhibitors from this chemotype bound to the PHD2 active site.¹¹ Although **ML228** has not been shown to act through PHD, molecular modeling of **ML228** was deemed an expeditious method for determining whether it could reasonably bind at this known binding site. Flexible superposition of an ensemble of 30 low energy structures of **ML228** with a structural hypothesis taken from a mutual flexible alignment of all ligands bound to the published PHD2 active site using SurflexSim²¹ gave a rather low shape based similarity score of 5.5 (normalized similarity on a 1–10 scale) (Figure 5). Generally, compounds with a higher likelihood to bind a similar protein site based on shape and surface considerations typically score above 7.0 in a SurflexSim shape-based similarity score. Furthermore, manual docking of **ML228** positioned to chelate iron in the active site revealed multiple negative steric interactions and a lack of favorable contacts. While not conclusive, molecular modeling suggests a low probability that **ML228** shares a similar mode of binding to PHD2 as those reported for analogs of **3**.

ML228 represents a novel chemotype available to the research community for the study of HIF activation and its therapeutic potential. Not only is the compound substantially different in structure from known HIF activators, **ML228** lacks the acidic functional group almost universally present in PHD inhibitors, which may be important for certain disease applications. **ML228** was demonstrated to potently activate HIF *in vitro* as well as its downstream target VEGF. Further biological evaluation of **ML228** is ongoing and will be reported in due course.

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17. Synthesis of ML228: (a) Compound 14. Ethanol was added dropwise to 2-cyanopyridine 13 (10 g, 96 mmol, 1.0 eq) and hydrazine hydrate (3.08g, 96.1 mmol, 1.00 eq) until a clear brown solution was obtained. The solution was allowed to sit at room temperature for 24 hours. Additional hydrazine hydrate (1.54 g, 48.0 mmol, 0.50 eq) was added, and the solution was allowed to sit for an additional 24 hours. The solvent was removed *in vacuo*, and the solid was washed with ether to give the title compound as an orange solid that was used without further purification. ES-MS [M + 1]⁺: 137.3. (b) Compound 15. Compound 14 (5.0 g, 37 mmol, 1.0 eq) was dissolved in ethanol (367 mL) and cooled to 0 °C. Ethyl benzoylformate (5.83 mL, 36.7 mmol, 1.00 eq) was added, and the reaction was allowed to warm to room temperature and stirred for 72 hours. The solvent was removed *in vacuo* to give a yellow solid that was used without further purification. ES-MS [M + 1]⁺: 251.2. (c) Compound 16. A dry, argon-purged vial was charged with compound 15 (180 mg, 0.72 mmol, 1.0 eq) and phosphorus oxychloride (1.8 mL) and subjected to microwave irradiation at 120 °C for 10 minutes. The resulting solution was added dropwise to cold, saturated NaHCO₃ and then extracted (3×) with cold CH₂Cl₂. The combined organics were filtered via phase separation, concentrated *in vacuo*, and further dried under vacuum to afford a yellow oil that was carried forward without purification. ES-MS [M + 1]⁺: 269.0. (d) ML228. Compound 16 (180 mg, 0.67 mmol, 1.0 eq) and 4-phenyl benzylamine (200 mg, 1.1 mmol, 1.6 eq) were dissolved in anhydrous THF (6.5 mL) and stirred at rt for 48 hours under argon. The reaction mixture was concentrated *in vacuo*. The concentrate was then dissolved in ethyl acetate, washed (2×) with 1N HCl, and washed (2×) with 1N NaOH. The aqueous layers were back-extracted (2×). The combined organics were dried (MgSO₄), filtered, and concentrated *in vacuo*. Purification by flash chromatography on silica gel afforded 115 mg (41%) of the title compound as a yellow solid: ¹H NMR (400 MHz, DMSO-d₆) δ 8.77 (d, *J* = 4.0 Hz, 1H), 8.31 (d, *J* = 7.9 Hz, 1H), 8.16 (dd, *J* = 5.9, 5.9 Hz, 1H), 7.97 (m, 1H), 7.75 (dd, *J* = 7.7, 1.7 Hz, 2H), 7.70 (m, 1H), 7.63-7.51 (m, 8H), 7.43 (dd, *J* = 7.6, 7.6 Hz, 2H), 7.33 (dd, *J* = 7.4, 7.4 Hz, 1H), 4.74 (d, *J* = 5.9 Hz, 2H), 4.14 (m, 1H); ES-MS [M + 1]⁺: 416.2.
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19. Targets where moderate binding displacement was noted (human unless otherwise noted): adrenergic α_{1D} (53%), adrenergic α_{2A} (57%), rat calcium channel L-type, dihydropyridine (56%), dopamine D₁ (54%), dopamine D₃ (67%), histamine H₁ (65%), melatonin MT₁ (51%), norepinephrine transporter (67%), opiate κ (71%), prostanoid EP₄ (53%).
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Figure 1.
Examples of PHD inhibitors

Figure 2.
HIF activator hit compounds

Figure 3.

Complete response curves for **ML228**. (A) HRE luciferase assay (B) Proteasome inhibition assay (C) HIF-1 α nuclear translocation high content imaging assay (D) RT-PCR VEGF transcription assay. The relative activity of **ML228** was compared to the neutral (DMSO, 0% activity) and the stimulator control (DFO 100 μ M, which equals to 100% activity).

Figure 4. Effects of metals on HRE luciferase activity of **ML228**. (A) medium only, EC₅₀ = 1.12 μM (B) 50 μM iron, EC₅₀ = 15.6 μM (C) 50 μM zinc, EC₅₀ = 5.01 μM. The relative activity of **ML228** is only compared to neutral control (DMSO) since the activity of DFO is reduced with the presence of iron and zinc.

Figure 5. Shape based molecular graphics aligned with the SurflexSim conformational ensemble illustrate the large dissimilarity in shape and volume of **ML228** (green surface) with the composite shape of the published PHD2 ligands.¹¹

Scheme 1.

Reagents and conditions: (a) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$, EtOH; (b) $\text{PhC(O)CO}_2\text{Et}$, EtOH; (c) POCl_3 , μw , 120°C ; (d) $\text{R}^1\text{R}^2\text{NH}$, THF

Table 1

SAR in isoxazole series

Entry	n	R	HRE luciferase EC ₅₀ (μM) ^a	HIF-1α nuclear translocation EC ₅₀ (μM) ^b	RT-PCR VEGF EC ₅₀ (μM) ^c
6	3	1-imidazolyl	28.4	7.72	15.8
10	4	OH	22.0	3.04	7.57
11	3	1-morpholinyl	23.9	20.0	28.0
12	1	cyclopropyl	24.9	16.6	21.5

^a HRE-luciferase assay in U2OS cells (n≥2)

^b High content imaging assay for nuclear translocation of HIF-1α (n≥2)

^c Real time PCR to evaluate VEGF transcription (n≥2)

Table 2

Initial SAR in triazine series

Entry	R	HRE luciferase EC ₅₀ (μM) ^a	HIF-1α nuclear translocation EC ₅₀ (μM) ^b	RT-PCR VEGF EC ₅₀ (μM) ^c
8		10.8	4.62	6.15
9		16.1	9.82	9.06
17		12.7	4.27	6.21
18		4.74	2.46	3.45
19		2.97	3.82	2.48
20		25.0	10.5	10.6
21		20.8	9.08	10.0
22		14.2	6.27	8.12
23		22.9	11.6	12.1
24		21.9	10.2	8.69

Entry	R	HRE luciferase EC ₅₀ (μM) ^a	HIF-1α nuclear translocation EC ₅₀ (μM) ^b	RT-PCR VEGF EC ₅₀ (μM) ^c
25		22.4	5.41	7.55
26		7.64	4.46	3.26
27		7.74	4.80	4.48
28		6.67	9.54	3.08
29		5.41	2.39	2.91
30		4.58	1.19	2.60

^a HRE-luciferase assay in U2OS cells (n≥3)

^b High content imaging assay for nuclear translocation of HIF-1α (n≥2)

^c Real time PCR to evaluate VEGF transcription (n≥2)

Table 3

SAR in analogs of **18** and **19**

Entry	R	HRE luciferase EC ₅₀ (μM) ^a	HIF-1α nuclear translocation EC ₅₀ (μM) ^b	RT-PCR VEGF EC ₅₀ (μM) ^c
31		4.17	2.06	2.55
32		6.73	1.20	1.88
33		29.5	7.67	8.38
34		3.63	9.82	8.72
35		17.5	9.84	9.60

^aHRE-luciferase assay in U2OS cells (n≥3)^bHigh content imaging assay for nuclear translocation of HIF-1α (n≥2)^cReal time PCR to evaluate VEGF transcription (n≥2)

Table 4

SAR in analogs of **27**

Entry	R	V	Q	HRE luciferase EC ₅₀ (μM) ^a	HIF-1α nuclear trans- location EC ₅₀ (μM) ^b	RT-PCR VEGF EC ₅₀ (μM) ^c
36	3,4-di-Me	CH	CH	2.96	2.30	1.85
37	3-Cl	CH	CH	2.33	1.49	2.77
38	4-OMe	CH	CH	4.74	3.73	3.67
39	3-OMe	CH	CH	4.15	6.27	3.92
40	4 ^t Bu	CH	CH	1.71	0.60	1.12
41	H	N	CH	26.0	5.02	4.35
42	H	CH	N	27.8	7.62	6.45
43	3-Ph	CH	CH	2.49	0.50	2.57
ML228	4-Ph	CH	CH	1.23	1.40	1.63

^a HRE-luciferase assay in U2OS cells (n≥3)

^b High content imaging assay for nuclear translocation of HIF-1α (n≥2)

^c Real time PCR to evaluate VEGF transcription (n≥2)

Table 5 Binding assay profile for **ML228**

Radioligand Binding Assay	Species	% Inhibition
Adenosine A ₃	human	80
Dopamine Transporter (DAT)	human	87
Opiate μ (OP3, MOP)	human	85
Potassium channel hERG	human	86
Serotonin (5-hydroxytryptamine) 5-HT _{2B}	human	92
Sodium Channel, site 2	rat	105