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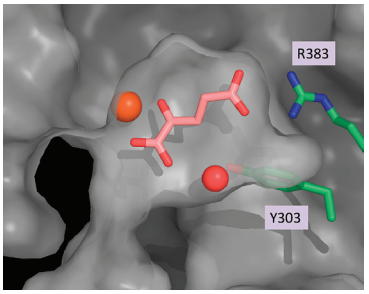
# Benzimidazole-2-pyrazole HIF Prolyl 4-Hydroxylase Inhibitors as Oral Erythropoietin Secretagogues

Mark D. Rosen,* Hariharan Venkatesan, Hillary M. Peltier, Scott D. Bembenek, Kimon C. Kanelakis, Lucy X. Zhao, Barry E. Leonard, Frances M. Hocutt, Xiaodong Wu, Heather L. Palomino, Theresa I. Brondstetter, Peter V. Haugh, Laurence Cagnon, Wen Yan, Lisa A. Liotta, Andrew Young, Tara Mirzadegan, Nigel P. Shankley, Terrance D. Barrett, and Michael H. Rabinowitz

Johnson & Johnson Pharmaceutical Research and Development, L.L.C, 3210 Merryfield Row, San Diego, California 92121, United States

**ABSTRACT** HIF prolyl 4-hydroxylases (PHD) are a family of enzymes that mediate key physiological responses to hypoxia by modulating the levels of hypoxia inducible factor 1-α (HIF1α). Certain benzimidazole-2-pyrazole carboxylates were discovered to be PHD2 inhibitors using ligand- and structure-based methods and found to be potent, orally efficacious stimulators of erythropoietin secretion in vivo.

**KEYWORDS** Prolyl 4-hydroxylase inhibitors, PHD inhibitors, hypoxia inducible factor, HIF, erythropoietin, epo, erythropoietin stimulating agents, anemia




HIF prolyl 4-hydroxylases (PHD1, PHD2, and PHD3) are a family of highly conserved, iron-containing, 2-oxoglutarate- (2OG) and dioxygen-dependent enzymes that play a crucial role in adaptation and survival during oxygen-deficient conditions.[1−3] Under normoxia, the transcription factor hypoxia inducible factor 1-α (HIF1α) is constitutively expressed and rapidly degraded, with a half-life of less than 5 min.[4] The degradation of HIF1α is regulated in part by PHD enzymes, which hydroxylate HIF1α proline residues 564 and 402, thereby enabling binding to the von Hippel-Lindau protein and subsequent proteasomic degradation.[5] Under a sufficiently low partial pressure of oxygen, the rate of PHD-mediated hydroxylation of HIF1α is lessened such that the production of HIF1α outpaces its degradation. The accumulated HIF1α then translocates to the nucleus, leading to the formation of a HIF1α-HIF1β heterodimer and the activation of genes involved in hypoxic responses such as erythropoietin (epo) secretion and erythropoiesis, anaerobic glycolysis, and angiogenesis.[6,7] Therefore, positive modulation of HIF transcriptional activity holds promise as a mode of treatment for a variety of debilitating ischemia-associated diseases, including anemia, myocardial infarction, stroke, and metabolic disorders, by conducting an orchestrated response to hypoxic challenge.[8,9]

Because of their key role in the regulation of HIF levels, inhibition of PHD enzymes is an attractive strategy by which to potentiate the transcriptional activity of HIF in a therapeutically relevant manner. Indeed, during the last several years, intense efforts to discover PHD inhibitors have been adumbrated primarily in the patent literature,[10] and to a much lesser degree in the peer-reviewed literature,[11−19] leading to four compounds entering clinical trials.[20,21] In this letter, we report our efforts in this area, which led to the discovery of novel PHD inhibitors that are potent, orally active erythropoietin secretagogues.

Screening of an internal compound library uncovered numerous strongly metal-ligating molecules that, upon closer investigation, appear to exert their inhibitory activity through iron sequestration and subsequent enzyme deactivation.[22] As this mechanism is unlikely to translate into a viable therapeutic method, we undertook an alternative approach to lead generation. A number of benzimidazole-2-glycinamides emerged as targets for chemical synthesis and subsequent evaluation as inhibitors, using a PHD2 enzymatic assay previously reported from this laboratory.[23] The synthesis of this initial set of potential inhibitors began with known or available benzimidazole-2-carboxylic acids **1a**−**h** (Scheme 1).[24] These acids were coupled to amino esters **2a**−**h**, which were then saponified to provide the corresponding benzimidazole acids **3a**−**h**.

Evaluation of compounds **3a**−**h** showed several to possess measurable inhibitory activity in our PHD2 enzymatic assay (Table 1). Dichloride analogue **3b** proved to be the most potent, with a $pIC_{50}$ value of 4.9. Representative members of this set were found to have low iron affinity in solution relative to literature reference compounds, suggesting that the observed inhibitory activity does not result from metal center depletion.[25] However, these particular benzimidazole inhibitors failed to significantly stimulate epo release from

**Received Date:** August 20, 2010
**Accepted Date:** September 29, 2010
**Published on Web Date:** October 05, 2010





DOI: 10.1021/ml100198y | *ACS Med. Chem. Lett.* **2010**, *1*, 526–529

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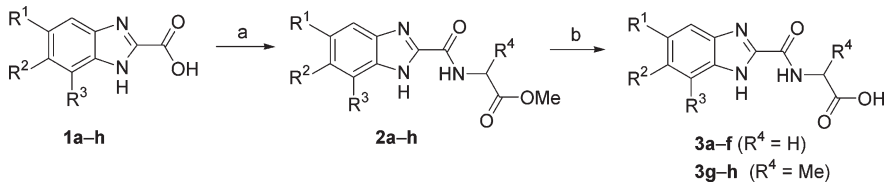
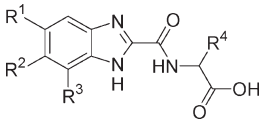
**Scheme 1.** Synthesis of Benzimidazole Amides **3a**−**h**[a]




[a] Reagents and conditions: (a) HATU, amino acid methyl ester hydrochloride, DIEA, DMF, 23 °C, 16 h. (b) LiOH, THF/$H_2O$, 23 °C, 2−16 h. For $R^{1-3}$ groups, see Table 1.

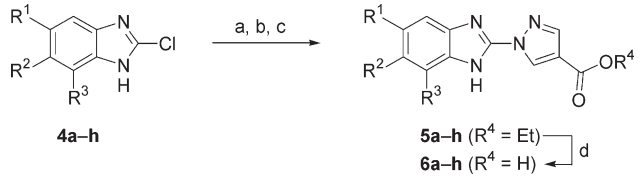
**Table 1.** PHD2 Enzyme Inhibition and Epo Release in Hep3B Cells for Compounds **3a**−**h**



| compd | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $pIC_{50}$[a] | epo release (%)[b] |
|---|---|---|---|---|---|---|
| **3a** | H | H | H | H | < 4 | < 20 |
| **3b** | Cl | Cl | H | H | 4.9 | < 20 |
| **3c** | Br | H | H | H | 4.6 | < 20 |
| **3d** | F | H | H | H | < 4 | < 20 |
| **3e** | OMe | H | H | H | 4.3 | < 20 |
| **3f** | $CF_3$ | H | $CF_3$ | H | 4.7 | < 20 |
| **3g** | $CF_3$ | H | $CF_3$ | (*S*)-Me | 4.3 | < 20 |
| **3h** | $CF_3$ | H | $CF_3$ | (*R*)-Me | 4.1 | < 20 |

[a] Negative logarithm of the concentration required to achieve 50% enzyme inhibition; the value is ±0.3 log units. [b] Percent increase in epo release from Hep3B cells at 100 μM concentration relative to positive control measured at 24 h. See the Supporting Information for assay details.

**Scheme 2.** Synthesis of Benzimidazole-2-pyrazoles **6a**−**h**[a]

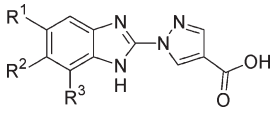



[a] Reagents and conditions: (a) SEMCl or MEMCl, DIEA or NaH, THF. (b) Ethyl pyrazole-4-carboxylate, $Cs_2CO_3$, DMF, 80 °C, 1−5 h. (c) 4 M HCl/dioxane, EtOH, reflux, 1 h. (d) LiOH, THF/$H_2O$. For $R^{1-3}$ groups, see Table 2.

Hep3B cells at concentrations up to 100 μM, while efforts to optimize the biopharmaceutical properties of the benzimidazole-2-carboxamide compounds proved challenging. As part of a larger search for suitable isosteric glycine replacement groups, we prepared a number of benzimidazole-2-pyrazoles as shown in Scheme 2.[26]

The synthesis of benzimidazole-2-pyrazoles **6a**−**h** began from known or available 2-chlorobenzimidazoles **4a**−**h**. Protection of the benzimidazole nitrogen atom as a MEM or SEM aminal facilitates displacement of the chloride atom by ethyl pyrazole-4-carboxylate in the presence of $Cs_2CO_3$.

**Table 2.** PHD2 Enzyme Inhibition, Epo Release, and HIF1α Accumulation for Compounds **6a**−**h**



| compd | $R^1$ | $R^2$ | $R^3$ | $pIC_{50}$[a] | epo (%)[b] | HIF1α (%)[c] |
|---|---|---|---|---|---|---|
| **6a** | H | H | H | 6.0 | < 20 | < 20 |
| **6b** | Br | H | H | 6.4 | 105 ± 6 | 70 ± 3 |
| **6c** | OMe | H | H | 6.4 | < 20 | < 20 |
| **6d** | Cl | F | H | 7.1 | 129 ± 11 | 72 ± 7 |
| **6e** | $OCF_3$ | H | H | 6.5 | 142 ± 10 | 63 ± 2 |
| **6f** | $OCF_3$ | H | Br | 6.8 | 56 ± 4 | 21 ± 1 |
| **6g** | $CF_3$ | Cl | H | 7.1 | 176 ± 7 | 92 ± 4 |
| **6h** | $CF_3$ | F | H | 7.0 | 28 ± 9 | 92 ± 5 |

[a] Negative logarithm of the concentration required to achieve 50% enzyme inhibition; the value is ±0.3 log units. [b] Percent increase in erythropoietin release from Hep3B cells at 100 μM concentration relative to positive control at 24 h. [c] Percent increase in HIF1α accumulation in Hep3B cells at 100 μM concentration relative to positive control measured at 24 h. See the Supporting Information for assay details.

Removal of the protective group under acidic conditions provided ethyl esters **5a**−**h**, which were saponified to afford the desired carboxylic acids **6a**−**h**.

Benzimidazole-2-pyrazoles **6a**−**e** proved to be potent inhibitors of PHD2 relative to the benzimidazole-2-carboxamides **3a**−**h** (Table 2). For example, compounds **6a**−**c** each show an increase in enzyme inhibitory activity of about 2 orders of magnitude relative to their direct comparators **3a**, **3c**, and **3e**, while several other compounds display $pIC_{50}$ values of 7.0 or more. As with the glycine amide compounds, selected members of this set also displayed low iron chelation ability in solution, indicating that the increased inhibitory activity was not a result of iron sequestration.[25] Of greater significance, these compounds effected a robust increase of epo secretion from Hep3B cells measured after 24 h of incubation. To demonstrate that the observed increase in epo secretion was a consequence of PHD inhibition, **6a**−**e** were tested in a HIF1α accumulation assay also using Hep3B cells, and compounds from this group were found to promote increases in HIF1α accumulation by as much as 92 ± 4% (e.g., **6g**, Table 2).

Having discovered compounds that potently inhibit PHD2 with concomitant increases in both HIF1α accumulation and



DOI: 10.1021/ml100198y | *ACS Med. Chem. Lett.* **2010**, *1*, 526–529

epo release in Hep3B cells, we evaluated **6g** and **6h** in an in vivo, orally dosed epo release model. Measured 6 h after a single oral dose of 100 $\mu$mol/kg, compounds **6g** and **6h** elicited increases in epo levels of 56- and 18-fold, respectively (Figure 1). This robust increase corresponds to plasma epo levels of 1410 ± 410 pg/mL versus the vehicle group value of 25 ± 8 pg/mL for the compound **6g** cohort and 1270 ± 760 pg/mL versus the vehicle group value of 72 ± 20 pg/mL for the **6h** cohort. For compound **6h**, this pharmacodynamic response occurred with 1 h plasma concentrations of 14 $\mu$M and 6 h concentrations of 0.1 $\mu$M.

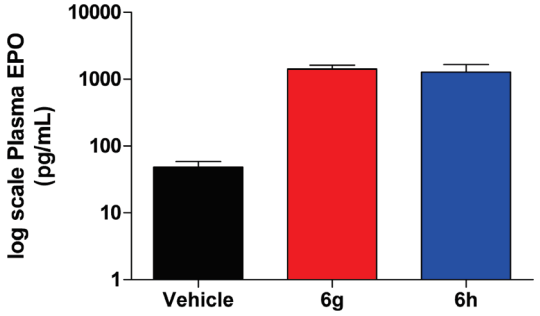



**Figure 1.** Epo levels vs vehicle for compounds **6g** and **6h** after a single oral dose of 100 $\mu$mol/kg in the mouse.

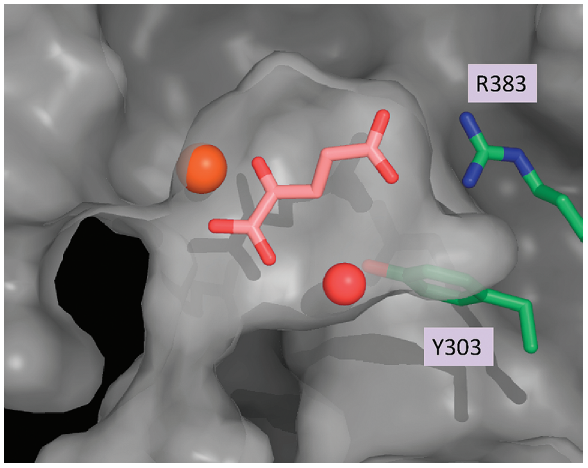



**Figure 2.** X-ray cocrystal structure of PHD2 and the endogenous ligand 2OG.

With these novel and structurally distinct PHD2 inhibitors in hand, we undertook structural studies to elucidate their mode of inhibition. Schofield, Syed, and co-workers, as well as Evodokimov and co-workers, have independently reported X-ray cocrystal structures of a truncated form of PHD2 with two structurally related isoquinoline inhibitors, both of which clearly mimic the endogenous cofactor 2OG in the active site of the enzyme.[27,28] To supplement these data for our own studies, we obtained the X-ray cocrystal structure of PHD2 with its natural ligand, 2OG (Figure 2).[29] In agreement with the previously published structures, the carboxylic acid group is clearly participating in a salt bridge interaction with R383, while the α-keto acid moiety is ligated to the iron atom in the active site. Furthermore, an ordered water cascade suggests the possibility that the α-ketocarboxylic acid moiety may be involved in a water-bridged interaction with Y303, analogous to the direct interaction observed between Y303 and the isoquinoline hydroxyl groups of the previously reported structures. Taken together, these X-ray crystal structures illustrate several key interactions between PHD2 and these known ligands.

In the case of benzimidazole glycine amide **3b** and pyrazole **6d**, the iron atom is engaged by the two $sp^2$ nitrogen atoms of the benzimidazole and pyrazole rings, respectively, while the carboxylic acid group appears well poised to interact with Arg383 (Figure 3).[29] Once again, the benzimidazole NH group is proximal to an ordered water molecule, suggesting a possible water-bridged interaction with Y303. These structures clearly demonstrate that compounds **3b** and **6d** act as 2OG mimetics and that they adopt a similar binding mode to that of the endogenous ligand.

In summary, we disclose the discovery and preliminary pharmacological profiling data for novel benzimidazole-based PHD2 inhibitors. The X-ray cocrystal structures of PHD2 with 2OG, **3b**, and **6d** are reported, and these structures elucidate key protein interactions and verify target engagement. Several compounds were identified that were efficacious in a series of assays, including biochemical enzyme inhibition, cell-based epo release and HIF1α accumulation, and in vivo

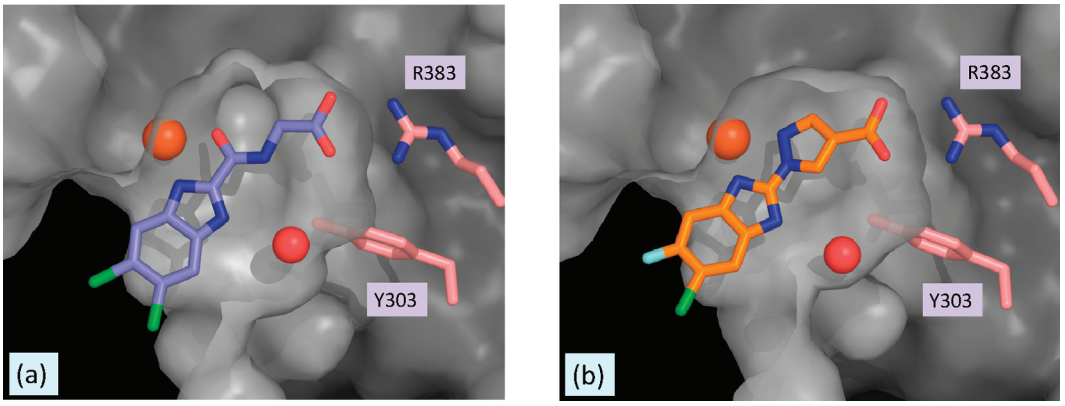



**Figure 3.** X-ray cocrystal structures of (a) PHD2/**3b** and (b) PHD2/**6d**.



DOI: 10.1021/ml100198y | *ACS Med. Chem. Lett.* **2010**, *1*, 526–529

epo release. This led to the discovery of compounds **6g** and **6h**, which were demonstrated to act as potent epo secretagogues when dosed orally in the mouse.

**SUPPORTING INFORMATION AVAILABLE** Experimental details for the synthesis and characterization of **3a**−**h** and **6a**−**h** as well as assay details. This material is available free of charge via the Internet at http://pubs.acs.org.

**ACKNOWLEDGMENT** We thank Dr. Jiejun Wu and Heather McAllister for analytical assistance.

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(29) The coordinates have been deposited with the RSCB Protein Data Bank. PHD2/2OG, PDB ID 3OUJ; PHD2/**3b**, 3OUI; and PHD2/**6d**, 3OUH.





DOI: 10.1021/ml100198y | *ACS Med. Chem. Lett.* **2010,** *1*, 526–529