



# [(4-Hydroxyl-benzo[4,5]thieno[3,2-c]pyridine-3-carbonyl)-amino]-acetic acid derivatives; HIF prolyl 4-hydroxylase inhibitors as oral erythropoietin secretagogues



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## ABSTRACT

A new series of PHD (HIF prolyl 4-hydroxylase) inhibitors was designed based on the X-ray co-crystal structure of FG-2216. Using a lead generation process, a series of [(4-Hydroxyl-benzo[4,5]thieno[3,2-c]pyridine-3-carbonyl)-amino]-acetic acid derivatives was developed as potent PHD2 inhibitors. This class of compounds also showed the ability to stabilize HIF- $\alpha$ , to stimulate EPO secretion in in vitro studies, and to increase hematocrit, red blood cell count, and hemoglobin levels in an animal efficacy study.

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Anemia is a common feature of advanced kidney disease and of cancer therapy that destroys the bone marrow. Recently, treatment of anemia has begun to focus on the application of small molecule erythropoiesis-stimulating agents (ESAs) to increase the blood's capacity for oxygen transport. The advent of orally available small-molecule ESAs such as hypoxia-inducible factor (HIF) stabilizers in the development of novel anti-anemia therapies has been revolutionary in terms of convenience and cost reduction of anemia treatments.<sup>1–3</sup>

Hypoxia-inducible factor (HIF) is a transcription factor and a key regulator of the body's local response to oxygen deprivation (hypoxia). Wang and Semenza revealed in 1992 that HIF binds to the hypoxia-responsive element (HRE) in the enhancer region of the erythropoietin gene. The following transcriptional activation was shown to regulate various physiological processes such as angiogenesis, erythropoiesis, energy utilization, vascular tone, apoptosis, and cellular proliferation.<sup>4,5</sup>

HIF itself is primarily regulated by prolyl hydroxylases (PHD1, 2, 3) as well as asparaginyl hydroxylase (FIH).

The HIF transcription factor consists of an HIF- $\alpha/\beta$  heterodimer that binds to hypoxia-response elements within the HIF target gene to control transcription.<sup>6</sup>

Under normoxic conditions, these PHDs specifically hydroxylate converse proline residues (Pro402 and Pro564 on human HIF- $\alpha$ ). These hydroxylated prolines of HIF- $\alpha$  create recognition sites for the von Hippel–Lindau protein (VHL) for proteasomal degradation. Thus, under adequate oxygen levels, the body continually expresses and degrades HIF- $\alpha$ .<sup>7</sup>

Stabilization of HIF- $\alpha$  by inhibition of PHDs could be a potential therapy for anemia, neuro-protection, ischemic disease including stroke, and complications of diabetes.<sup>8–11</sup> For these reasons, many small molecule drug candidates have been proposed as PHD2 inhibitors with a wide range of potencies measured from the discovery stages to clinical trial stages, as shown in Figure 1.<sup>12,13</sup>

One of the pioneers in the field of commercial research on prolyl hydroxylase inhibition is Fibrogen, which runs clinical trials for the therapeutic use of HIF stabilizers in the most advanced stages. FG-2216, the lead drug candidate, has completed Phase II trials, while the second generation FG-4592 is currently enrolling for Phase IIa and IIb trials in the USA.<sup>14–16</sup>

GlaxoSmithKline has been testing its investigational HIF prolyl hydroxylase inhibitor GSK1278863 for the treatment of anemia since 2008. It has completed several Phase I studies in the USA and is currently running Phase II studies with pre-dialysis and hemodialysis-dependent patients in Australia, New Zealand, India, and the Russian Federation. Recently, the drug candidate GSK360A entered the stage of a pre-clinical development program. GSK360A was reported to mediate cardioprotective effects and to raise EPO and Hb levels in rats.

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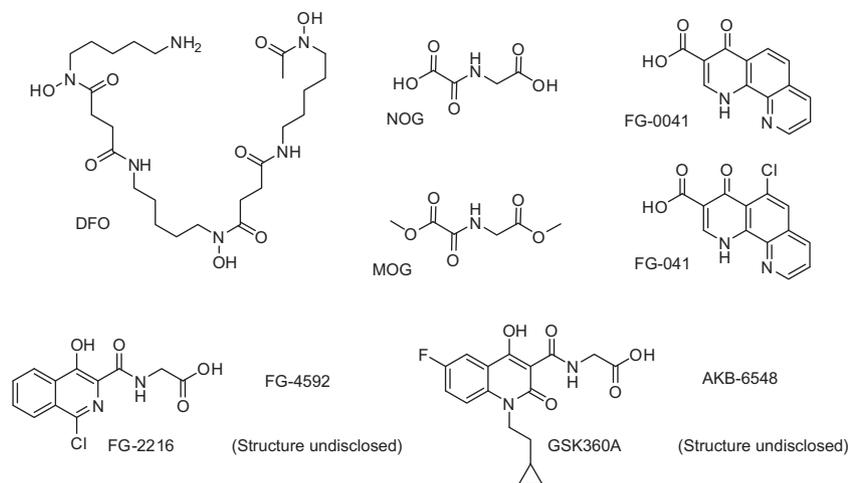


Figure 1. Examples of the known PHD2 inhibitors.

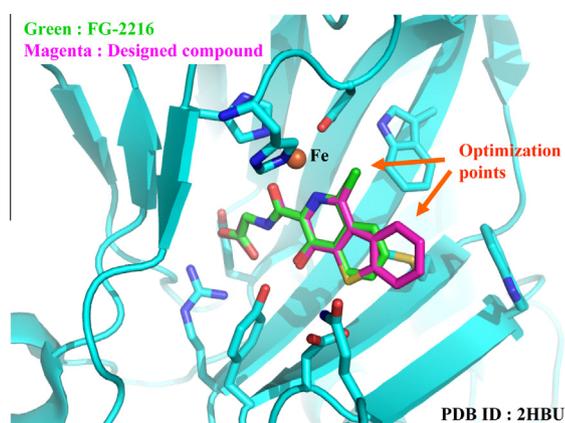


Figure 2. The known binding mode of FG-2216 with the catalytically active domain of human PHD2 (PDB: 2HBU) and its additional optimization points to increased hydrophobic interactions; the active site is small and narrow, so the testing compounds of the new class were designed to fill the empty spaces with hydrophobic functional groups.

Table 1  
Inhibitory activities of compounds designed to increase additional interactions against the PHD2 enzyme

Compounds	X	R <sup>1</sup>	PHD2 IC <sub>50</sub> (μM)	Stdev.
FG-2216	–	–	3.9	0.1
<b>1</b>	O	H	45.5	6.4
<b>2</b>	S	H	8.6	0.8
<b>3</b>	S	Me	1.9	0.1
<b>4</b>	S	Cl	1.5	0.1
<b>5</b>	S	CN	0.7	0.1

Akebia Therapeutics, Inc., founded in 2007, has in-licensed pre-clinical programs on the HIF prolyl hydroxylase inhibitor (AKB-6548) at Proctor & Gamble. Akebia is currently recruiting CKD (Chronic Kidney Disease) patients for Phase II trials to test the change in Hb levels following once-daily administration.

In 2006, two independent working groups succeeded in co-crystallizing a catalytically active domain of human PHD2 with FG-2216 or its derivative and analyzed the purified complexes by X-ray crystallography.<sup>17</sup> The solved structures provided a new foundation for the understanding of the molecular mechanism of PHD catalysis and inhibition and offered new perspectives for the development of novel, more specific PHD inhibitors (Fig. 2).

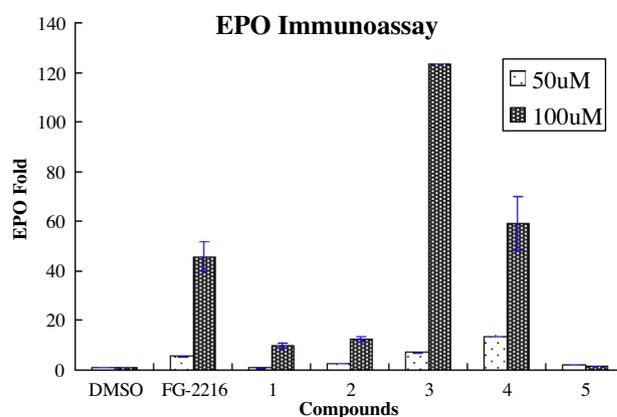
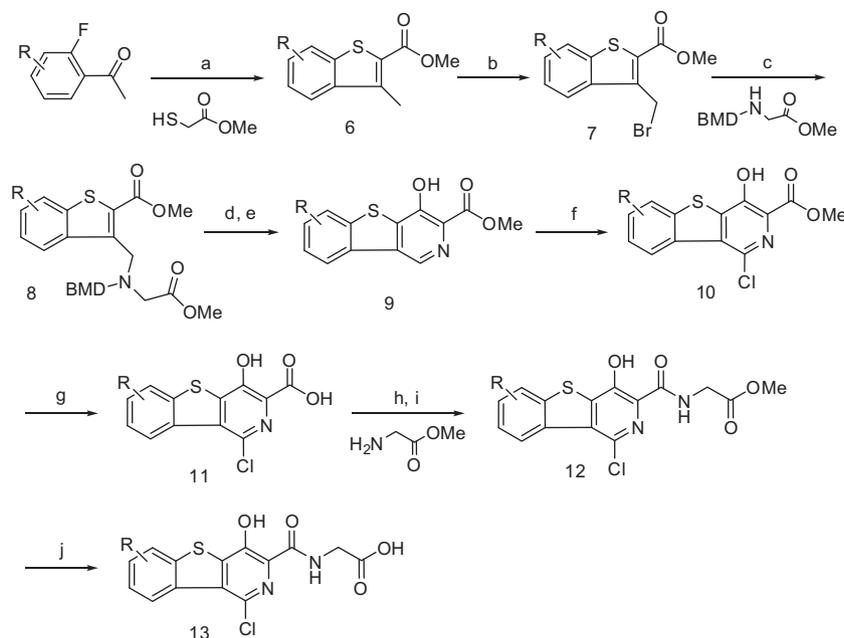


Figure 3. EPO secretion by PHD2 inhibition after 24hr from each compound treatment in Hep3B cell.

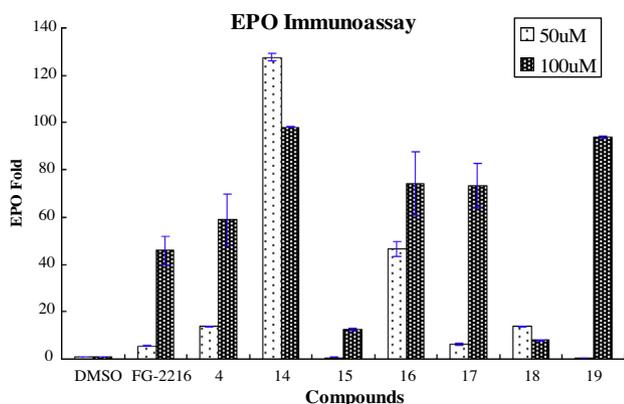
Table 2  
Inhibitory activities of the [(benzo[4,5]thieno[3,2-c]pyridine-3-carbonyl)-amino]-acetic acid analogs against the PHD2 enzyme

Compounds	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	R <sup>5</sup>	PHD2 IC <sub>50</sub> (μM)	SD
FG-2216					3.9	0.1
<b>4</b>	H	H	H	H	1.5	0.1
<b>14</b>	H	H	N(Et) <sub>2</sub>	H	1.8	0.1
<b>15</b>	F	H	H	H	4.1	0.2
<b>16</b>	H	F	H	H	2.8	0.2
<b>17</b>	H	H	H	F	1.7	0.1
<b>18</b>	H	H	OH	H	0.5	0.0
<b>19</b>	H	OH	H	H	1.3	0.1

In the binding observed, we can find two optimization points that are well suited to fill the predominantly hydrophobic pockets. Additional interactions of these pockets have not been reported in known co-crystal structures. The optimization study predicted new analogs of compounds **1–5** and these were synthesized to test these predictions. The synthesized analogs were tested for their activities of PHD2 inhibition using a fluorescence polarization method.<sup>18</sup> These structure activity relationships for 'X' and 'R<sup>1</sup>' are highlighted in Table 1. When 'X' is a sulfur atom rather than an oxygen atom, the inhibitory activity of PHD2 is substantially increased, and compounds **3–4** show higher activities than FG-2216 due to additional interactions, as expected.



**Scheme 1.** Reagents and conditions: (a) NaH/THF, 0 °C, then reflux, 45–57%; (b) NBS, (PhCO<sub>2</sub>)<sub>2</sub>/dichloromethane, reflux, 74–85%; (c) K<sub>2</sub>CO<sub>3</sub>/dichloromethane, reflux, 70–86%; (d) *t*-BuOK/THF, –20 °C; (e) SOCl<sub>2</sub>/dichloromethane, 0 °C then 40 °C, 25–46% for 2 steps; (f) NCS, (PhCO<sub>2</sub>)<sub>2</sub>/dichloromethane, reflux, 65–72%; (g) LiOH/H<sub>2</sub>O, MeOH, THF, 40 °C, 85–90%; (h) oxalyl chloride/dichloromethane, rt; (i) Et<sub>3</sub>N/dichloromethane, rt, 61–75% for 2 steps; (j) LiOH/H<sub>2</sub>O, MeOH, THF, 40 °C, 72–80%.

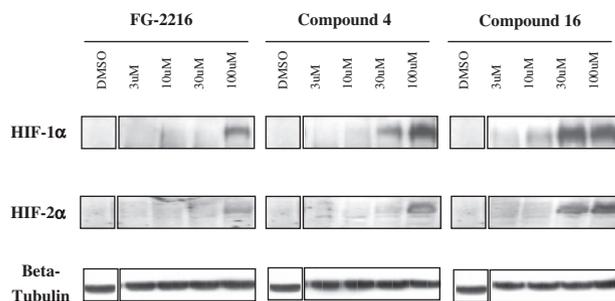


**Figure 4.** EPO secretion by PHD2 inhibition after 24 hr from each compound 4 analogs treatment in Hep3B cell.

The expression of EPO due to PHD2 inhibition was determined using an EPO ELISA method<sup>19</sup> in Hep3B cells. The EPO expression results for each compound are presented in Figure 3.

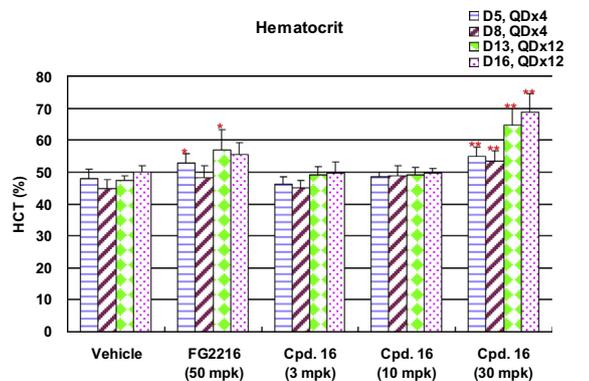
**Table 3**  
Metabolic stability study and Mouse PK study for three compounds

Item	Sub-item/Compound	Compound 4		Compound 14		Compound 16	
Metabolic stability, remaining % at 60 min	Method/Species	Mouse	Human	Mouse	Human	Mouse	Human
	Microsomal	68.60%	118.60%	81.70%	78.50%	84.40%	75.90%
	S9 fraction	125.70%	100.90%	85.00%	77.30%	106.70%	126.50%
Mouse PK	Parameters/Route	IV	PO	IV	PO	IV	PO
	Dose	4	20	4	20	4	20
	T <sub>max</sub> (h)	–	0.5	–	0.5	–	0.17
	C <sub>max</sub> (μg/mL)	–	15.1	–	3.9	–	39.6
	AUC <sub>0–t</sub> (μg h/mL)	17.0	53.7	6.3	13.7	31.1	90.1
	T <sub>1/2</sub> (h)	2.79	–	3.4	–	3.23	–
	CL (mL/h kg)	235.3	–	635.8	–	128.2	–
	V <sub>ss</sub> (mL/kg)	503.4	–	831.6	–	401.6	–
	F (%)	63.2	–	43.6	–	57.7	–

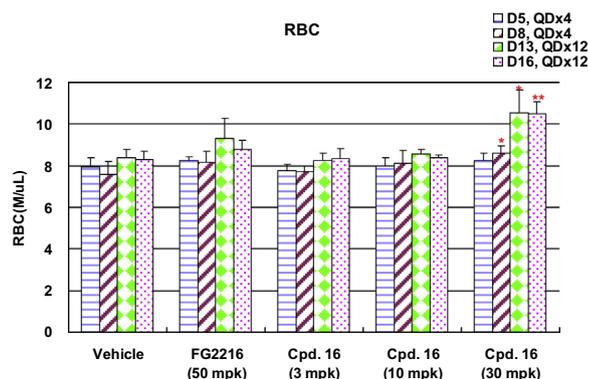


**Figure 5.** HIF- $\alpha$  stabilization in Hep3B cells by PHD2 inhibition due to compound 4 and 16 after 24 h incubation.

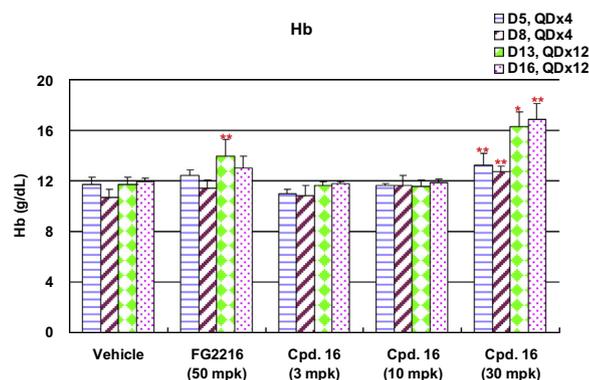
The initial SAR (from Table 1 and Fig. 3) of the synthesized analogs revealed that compound 3 at 100  $\mu$ M resulted in the greatest EPO secretion; however, compound 4 is a more desirable candidate since it showed greater EPO secretion at 50  $\mu$ M. We enabled SAR exploration of novel classes of PHD2 inhibitors like compound 4 by successfully developing an optimal synthetic route and preparing analogs of various 'R' according to Scheme 1.



\*; P<0.05; \*\*; P<0.01 vs. vehicle of each blood sampling time; these



\*; P<0.05; \*\*; P<0.01 vs. vehicle of each blood sampling time



\*; P<0.05; \*\*; P<0.01 vs. vehicle at each blood sampling time

**Figure 6.** Efficacy study by PHD2 inhibitions of FG-2216 and compound **16** in a mouse model.

Using this synthetic route, the structure activity relationship for phenyl group substitutions is highlighted in Table 2.

The expression of EPO following PHD2 inhibition by the new analogs was determined using an EPO ELISA method and Hep3B cells. The EPO expression results for each compound are presented in Figure 4.

Secondary SAR (Table 2 and Fig. 4) of the new synthesized analogs revealed that treatment with compounds **14** and **16** resulted in good EPO secretion at 50  $\mu$ M and 100  $\mu$ M. A significant increase in EPO secretion was seen with the introduction of diethylamine to R<sup>4</sup> (compound **14**); this analog showed a nine-fold improvement in EPO secretion compared to FG-2216 at 50  $\mu$ M. However, compound **14** did not show a continuous increase in EPO secretion until it was added at a high concentration, while EPO secretion was decreased at 100  $\mu$ M. Further optimization of compound **14** was achieved as shown by compound **16**, which demonstrated a greater than 3.4-fold and 1.6-fold increase in potency compared to FG-2216 at 50  $\mu$ M and 100  $\mu$ M, respectively. Compound **16** also showed no diminution of EPO secretion as the concentration of the inhibitor increased, unlike the case for compound **14**. Therefore, compound **16** was considered to be a more ideal candidate than compound **14**. Additionally, we checked the HIF stabilization effects of compound **4** and compound **16** for HIF-1 $\alpha$  and HIF-2 $\alpha$  in Hep3B cells, and confirmed superior effects of new synthesized analogs compared with FG-2216 (Fig. 5).

Three compounds (compounds **4**, **14**, and **16**) were also compared for metabolic stability and mouse PK following exposure in order to select a reasonable candidate. The results are presented in Table 3.

All three compounds are quite metabolically stable in mouse and human species. The bioavailabilities and half-lives of the three compounds were very similar. However, among the three compounds, compound **16** showed the best activity with respect to C<sub>max</sub> and AUC in the mouse PK studies.

Ultimately, compound **16** was selected as the final candidate for an animal study. The efficacy study was designed to perform daily administration for 4 days (QDx4) and 12 days (QDx12) in normal ICR mice (male, 6 weeks old) like Table 4. The mice were divided into 4 groups by blood sampling times (D5, D8, D13, and D16) with respect to treatment with vehicle, FG-2216, or compound **16**. Compound **16** was also used at three doses (3 mpk, 10 mpk, and 30 mpk) in this efficacy study. The results for each group are presented in Figure 6.

The PD studies of FG-2216 and compound **16** revealed that compound **16** had superior efficacy at a lower dose of 30 mpk than did FG-2216 at 50 mpk. The hematocrit, red blood cell counts, and hemoglobin levels, which all participate in erythropoiesis, were all increased by 30 mpk treatment with compound **16**. A long-term treatment of 12 days was the most efficacious method for treating anemia with compound **16**, compared to shorter-term treatments.

In summary, we designed a new series of PHD2 inhibitors based on the X-ray co-crystal structure of FG-2216. A lead generation process was used to develop a series of [(4-Hydroxyl-benzo[4,5]thieno[3,2-c]pyridine-3-carbonyl)-amino]-acetic acid derivatives as potent PHD2 inhibitors. In addition, this class of compounds showed the ability to stabilize HIF- $\alpha$ , to stimulate EPO secretion in in vitro studies, and to increase hematocrit, red blood cell counts, and hemoglobin levels in an animal efficacy study. Currently, pre-clinical studies are in progress to determine the therapeutic potential of the selected analogs.

**Table 4**  
Administration schedule of efficacy study

Groups/day	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16
D5, QDx4	↑	↑	↑	↑	✓											
D8, QDx4	↑	↑	↑	↑			✓									
D13, QDx12	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	✓			
D16, QDx12	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑				✓

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2013.08.067>.

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