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2-Oxoglutarate analogue inhibitors of prolyl hydroxylase domain 2

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ABSTRACT

Analogues of the 2-oxoglutarate cosubstrate of the human oxygen sensing enzyme prolyl hydroxylase domain 2 (PHD2) with variations in the potential iron-chelating group were screened as inhibitors and for binding (using non-denaturing electrospray ionization mass spectrometry) to PHD2.

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Many human genes are hypoxically regulated, including those encoding for vascular endothelial growth factor and erythropoietin. At the transcriptional level, the hypoxic response is mediated by hypoxia-inducible factor (HIF), an α,β-heterodimeric protein complex.[1] Levels of its HIF-α subunit are regulated by oxygen availability. Under normoxic conditions *trans*-4-hydroxylation of prolyl residues (Pro402, Pro564) in HIF-α is catalyzed by HIF prolyl hydroxylases (PHD1-3) that are Fe(II) and 2-oxoglutarate (2OG) dependent oxygenases.[2–6] Prolyl-hydroxylation enables recognition of HIF-α by the von Hippel-Lindau tumour suppressor protein (pVHL) which in turn causes ubiquitinylation and proteasomal degradation of HIF.[7] When oxygen becomes limiting, the HIF-α subunit accumulates and dimerizes with HIF-β to enable the hypoxic response.

PHD2 is thought to be the most important of the PHDs for the hypoxic response in normal tissues. Inhibition of PHD2 is suggested to be useful in treatment of anaemia and ischemic disease.[8] Small molecule inhibitors of the PHDs and other human 2OG oxygenases may also be useful for functional assignment work and biophysical studies. Most reported 2OG oxygenase inhibitors are 2OG analogues (or at least compete with 2OG for binding). Here, we report the evaluation of simple 2OG analogues with variations in the potential iron-chelating group as PHD2 inhibitors and studies on their binding affinity employing non-denaturing mass spectrometry.

N-Oxalylglycine (NOG, **1**), which was initially used as a collagen prolyl hydroxylase (CPH) inhibitor,[9] is a 2OG analogue that inhibits most 2OG oxygenases. Further 2OG analogues having simple scaffolds with iron-chelating groups were identified as potential 2OG competitive inhibitors of PHD2 (Fig. 1). A previous report showed that the NOG analogue thionoamide, which has a sulfur instead of oxygen at the C-2 position, did not efficiently inhibit PHD2.[10] Together with structural analyses,[11] this result implies that the 2-oxo, or equivalent, group has an important role in iron chelation. Modification of the 2OG 1-carboxylate to give potent inhibitors has been achieved in the form of aromatic heterocycles and phenols that chelate the active site Fe(II) in a bidentate manner via a side-chain carbonyl oxygen and an aromatic nitrogen or phenolic-oxygen.[12–18]

Because the 2OG/NOG binding site of PHD2 is relatively narrow it may be difficult to achieve selectivity via binding in this pocket.[19] It was considered that C-1 ester/amide derivatives may provide a way to introduce a functionality that could enable selective inhibition, whilst still maintaining bidentate iron chelation (**2**–**7**). Such compounds could also be used to test the importance of the 1-carboxylate functionality in the inhibition properties of NOG analogues. Further, based on the structure of the known inhibitor dealanylalahopcin,[20] it was proposed that simple hydroxamic acids (**8, 9**) may

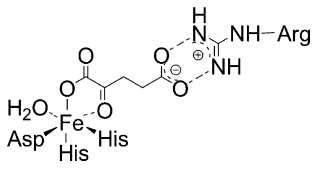

Figure 1. Proposed binding mode of 2OG to PHD2 via bidentate chelation of the active site Fe(II) and a formation of a salt-bridge with Arg383.[21]

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be inhibitors. Although NOG likely chelates the active site Fe(II) of PHD2 via formation of a five-membered chelate ring, we envisaged that malonyl derivatives could form a six-membered chelate ring, hence these were also prepared (**10**–**14**). A tricarbonyl functionality was also proposed to allow iron chelation and consequent inhibition of PHD2 (**15**). Furthermore, substitution of the carboxylic acid of the glycine moiety of NOG (**1**) by a methyl ester (**16**) was chosen to investigate the importance of this carboxylic acid in inhibition.

Initially, the 2OG analogues **2**–**16** were prepared and screened for inhibition against the catalytic domain of PHD2 (residues 181–426) employing a time resolved fluorescence resonance energy transfer (FRET) assay.[6] Amongst the designed 2OG analogues **2**–**16**, only **2**, **3**, **8**, **10**, **13** and **15** significantly inhibited PHD2 at 400 μM. The other analogues displayed weaker (<50% reduction of initial rate) inhibition activity at 400 μM or 1 mM. IC_{50} values were determined for these after a 10 min preincubation. As reported, NOG (**1**) was a relatively good PHD2 inhibitor (IC_{50} 26 μM).[22]

The C-1 mono-methyl (**2**) and C-1 mono-ethyl (**3**) esters had IC_{50} values of 95 μM and 78 μM, respectively, while the C-5 mono-methyl ester **16** displayed only weak inhibition activity (IC_{50} > 1 mM). Esters **2** and **3** were subsequently found to undergo hydrolysis to give NOG **1** during the assay (see below). The lack of inhibition by **16** is in agreement with crystallographic data revealing that the formation of a salt-bridge between the glycine side-chain carboxylic acid of the inhibitor and Arg383 contributes to the formation of a complex with NOG.[21]

In contrast to NOG **1**, none of the *N*-oxalyl amide or diamide compounds **4**–**7** displayed any significant inhibitory potency at 1 mM (Table 1). These results illustrate the importance of the α-ketoacid functionality in the binding by NOG, and suggest that the oxalyl diamide scaffold is likely an unsuitable target for further exploration.

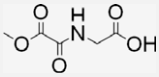
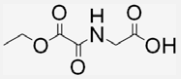
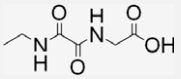
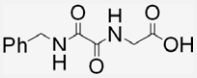
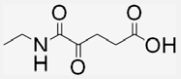
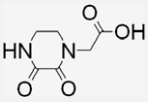
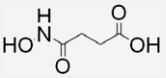
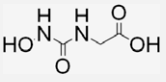
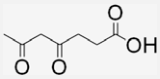
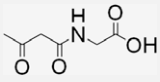
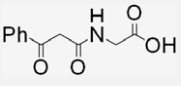
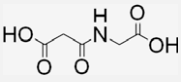
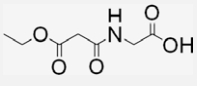
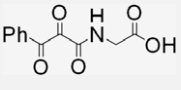
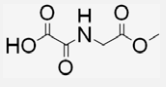
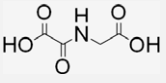
Among the new inhibitors screened, 4,6-dioxoheptanoic acid **10** is of interest. Whilst its amide analogue **11** showed weak activity (IC_{50} > 1 mM), **10** was a relatively potent inhibitor (IC_{50} 18 μM, Table 1). The aromatic malonyl derivative **12** was found to be only a weak inhibitor of PHD2 (IC_{50} > 1 mM). Malonic acid **13** and its ethyl ester **14** exhibited different inhibition potencies; while the former inhibited with an IC_{50} of 398 μM, the latter was not active (IC_{50} > 1 mM). Tricarbonyl compound **15** inhibited PHD2 with an IC_{50} of 538 μM (Table 1); however, like **2** and **3** underwent non-enzyme mediated decomposition to give NOG **1**. Hydroxamic acid **8**, that was reported to inhibit the 2OG oxygenase AlkB,[23] inhibited PHD2 with an IC_{50} of 94 μM; in contrast its amide analogue **9** showed only very weak inhibition activity at 1 mM (IC_{50} > 1 mM, Table 1).

The analogues were then analysed for binding to the catalytic domain of PHD2 employing non-denaturing electrospray ionization mass spectrometry (ESI-MS).[13,24] C-1 Monoalkyl esters of NOG **2**–**3** were observed to bind to PHD2, but the observed species both had a molecular mass that corresponded to NOG **1** (Supplementary Fig. 1). This result is explained by the non-enzymatic hydrolysis of **2**–**3** to provide the inhibitor NOG **1**.[22] In contrast, the C-5 mono-methyl ester **16** did not form complex with PHD2 (Supplementary Fig. 1), indicating the importance of the C-5 carboxylic acid functionality of the glycine side-chain for the binding. The observation that **2** and **3** are hydrolysed, but not **16**, presumably reflect the likely enhanced reactivity of the C-1 esters of **2** and **3**. This result illustrates the utility of protein ESI-MS in detecting the reaction of inhibitors under incubation conditions.

The analogues **4**–**6** did not display any affinity to form complexes with PHD2 (Supplementary Fig. 2), consistent with their lack of inhibition.

Recently, Desseyn et al. have studied the structure of oxamides, employing ab initio calculations, X-ray crystallography, NMR spec-

Table 1
Inhibition data for PHD2 using the FRET turnover assay (**2**, **3** and **15** underwent reaction to **1** under the assay condition)

	Structure	IC_{50} (μM)
2 (**1**)		95
3 (**1**)		78
4		>1000
5		>1000
6		>1000
7		>1000
8		94
9		>1000
10		18
11		>1000
12		>1000
13		398
14		>1000
15 (**1**)		538
16		>1000
1		26

troscopy, thermal analysis and Raman spectroscopy.[25] The combined use of these techniques revealed that oxamides preferentially possess a *trans* geometry because of intramolecular hydrogen bonding. Crystallographic analyses also revealed that when *N*-cyclopentyl-*N*-(thiazol-2-yl)oxalamide forms a complex with methionine aminopeptidase and a Co(II) ion, its oxamide moiety possesses a *trans* geometry when chelates the Co(II) ion.[26] Thus, the lack of observed binding to PHD2 of **4** and **5** may, at least in part, be due to the prefered *trans* geometry of the diamide moiety in these two compounds. This issue does not arise with *N*-oxalylacids or 2-oxoacids. A cyclic diamide **7** also did not form a complex with PHD2 (Supplementary Fig. 2); the steric demand of **7** may play a role in its lack of observed binding.

Hydroxamic acids **8** and **9** displayed different affinities for binding to PHD2 by ESI-MS. Whilst **8** displayed a significant ability to form a complex PHD2.Fe.**8** at 28243 Da, the latter did not bind (Supplementary Fig. 2). These observations are in agreement with the turnover inhibition data showing that hydroxamic acid **8**, unlike **9**, inhibited PHD2.

The ESI-MS results also revealed that 4,6-dioxoheptanoic acid **10** formed a strong complex at 28268 Da corresponding to PHD2.Fe.**10**, while its analogue **11**, containing an amide instead of a 4-carbonyl group, displayed a much reduced ability to form a PHD2.Fe.**11** complex (Fig. 2). The ability of **10** to form a strong complex (by ESI-MS) and inhibit PHD2 can in part be explained by its acidic C-5 methylene, that facilitates bidentate iron chelation in an analogous manner to 2OG (Fig. 1), but via a 6-, rather than a 5-, membered chelate ring. Malonic acid derivatives **11**–**13** also formed moderately stable complexes with PHD2. However, the ethyl ester **14** formed only a very weak complex (Fig. 2). Notably, the ESI-MS studies revealed that the tricarbonyl compound **15** underwent ferric iron-mediated cleavage to produce NOG in the absence of PHD2 (see Ref. 27 for detailed studies on this reaction), exemplifying the use of this technique for monitoring the reaction.[27]

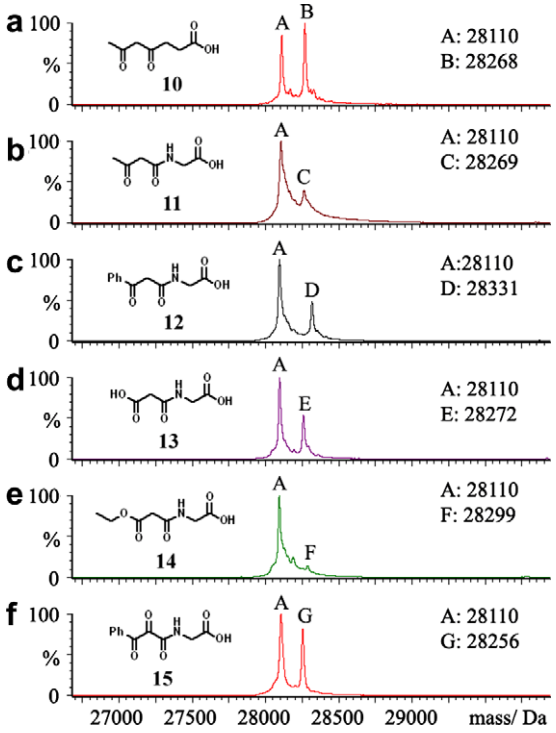


Figure 2. Non-denaturing ESI-MS spectra for PHD2 in the presence of equimolar amounts of $FeSO_4$ and inhibitors (a) **10**; (b) **11**; (c) **12**; (d) **13**; (e) **14**; (f) **15**. Peaks present: (A) PHD2.Fe; (B) PHD2.Fe.**10**; (C) PHD2.Fe.**11**; (D) PHD2.Fe.**12**; (E) PHD2.Fe.**13**; (F) PHD2.Fe.**14**; (G) PHD2.Fe.**1**.

Overall, several new types of 2OG analogues have been evaluated as inhibitors of PHD2. Among the newly identified inhibitors, two new scaffolds may be promising for further development into more potent inhibitors. 4,6-Dioxoheptanoic acid **10** and hydroxamic acid **8** were shown to efficiently inhibit PHD2, whilst the other tested 2OG analogues displayed weaker or no inhibition activity against PHD2. In particular **10** could be modified by C-5 derivatisation. Binding studies employing non-denaturing ESI-MS analysis provided further evidence that the more efficient inhibitors (**1**, **2**, **3**, **8**, **10**) formed strong complexes with PHD2, whereas weak inhibitors showed a decreased binding affinity. Thus, the results also further validate the use of non-denaturing ESI-MS for assuming binding affinities of small molecules to enzymes.[23,28]

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Supplementary data

Supplementary data (mass spectra and synthetic procedures) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.09.005.

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