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Dual-Action Inhibitors of HIF Prolyl Hydroxylases That Induce Binding of a Second Iron Ion

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Abstract

Inhibition of the hypoxia-inducible factor (HIF) prolyl-hydroxylases (PHD or EGLN enzymes) is of interest for the treatment of anemia and ischemia-related diseases. Most PHD inhibitors work by binding to the single ferrous ion and competing with 2-oxoglutarate (2OG) co-substrate for binding at the PHD active site. Non-specific iron chelators also inhibit the PHDs, both *in vitro* and in cells. We report the identification of dual action PHD inhibitors, which bind to the active site iron and also induce the binding of a second iron ion at the active site. Following analysis of small-molecule iron complexes and application of non-denaturing protein mass spectrometry to assess PHD2-iron-inhibitor stoichiometry, selected diacylhydrazines were identified as PHD2 inhibitors that induce the binding of a second iron ion. Some compounds were shown to inhibit the HIF hydroxylases in human hepatoma and renal carcinoma cell lines.

Keywords

Hypoxia inducible factor; HIF; hydroxylase; oxygenase inhibitor; 2-oxoglutarate; prolyl hydroxylase; oxygenase

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Supporting Information Available: Additional experimental information, including synthetic procedures and full characterization of all new compounds.

Introduction

In humans and other animals, the effects of limiting oxygen are counteracted by the hypoxic response, which is regulated by the oxygen-dependent post-translational hydroxylation (Fig. 1A) of the α -subunit of hypoxia inducible factor (HIF). Hydroxylation of either of two prolyl residues (Pro402 at the *N*-terminal oxygen-dependent degradation domain (NODD) and Pro564 at the *C*-terminal oxygen-dependent degradation domain (CODD) in human HIF-1 α) signals for the degradation of HIF-1 α via the ubiquitin-proteasome pathway. When oxygen availability becomes limiting, HIF prolyl-hydroxylase activity is reduced, resulting in accumulation of HIF-1 α , which dimerizes with constitutively expressed HIF-1 β to stimulate expression of genes with hypoxia-responsive element (HRE)-containing promoters. In humans, there are three isoforms of the HIF prolyl hydroxylases (PHD1-3 or EGLN1-3 enzymes). Additionally, factor inhibiting HIF (FIH) catalyzes asparaginyl hydroxylation of HIF-1 α (Asn803) in the *C*-terminal transactivation domain (CAD) to reduce its interaction with the transcriptional coactivator p300 in an oxygen-dependent manner. PHD2 (EGLN1) is probably the most important of the HIF hydroxylases for oxygen sensing in healthy human tissues (for reviews see¹⁻⁵). Both the PHD and FIH enzymes are proposed to contribute to the oxygen sensing capacity of the HIF system and belong to the large family of Fe(II) and 2-oxoglutarate (2OG) dependent oxygenases (2OG oxygenases⁶⁻⁸), which share common structural and mechanistic features (Fig. 1A and 1B).

Inhibition of the PHDs has potential for the treatment of anemia, ischemia-related diseases, and other diseases^{9, 10} by enabling a range of cellular and systemic responses that enhance oxygen delivery or reduce oxygen demand. Early studies demonstrated that iron chelators, such as deferoxamine (DFO, Fig. 1C), and transition metals ions, such as Co(II), also induce the hypoxic response,^{11, 12} with the latter likely to act (at least in part) via competition with Fe(II) for binding to the active sites of the HIF hydroxylases. Several transition metal ions have also been shown to act as 2OG oxygenase inhibitors.^{11,13}

Following earlier studies on procollagen prolyl hydroxylases, small-molecule inhibitors of the PHDs were identified,^{10, 14-21} most of which compete with the 2OG co-substrate for binding to the enzyme active site (for review, see²²). Most, if not all, of the reported compounds that inhibit 2OG oxygenases bind to the single active site metal in a bidentate manner as observed by crystallographic analyses.^{6, 7, 15, 23-25} Representative examples of 2OG-competitive inhibitors displaying bidentate iron chelation include the generic 2OG oxygenase inhibitor *N*-oxalyl glycine (NOG, its cell-penetrating ester form is dimethyl oxalyl glycine, DMOG), as well as bicyclic inhibitors such as 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido)acetic acid, BIQ²⁶ (Fig. 1C).

Overall, previous studies have demonstrated that PHD inhibition can be achieved by limiting iron availability (e.g. as with the iron chelator DFO) or by specific binding of inhibitors to the active sites of HIF hydroxylases. It is unclear whether a selective and complete inhibition of all HIF hydroxylases is desirable to better mimic the 'natural' hypoxic response, due to the potential involvement of oxygenases other than the HIF hydroxylases and the lack of understanding of the precise roles of individual PHD isoforms and FIH. Indeed, relatively non-specific compounds may better mimic the 'natural' hypoxic response.

We therefore aimed to develop inhibitors that bind to the PHD active sites and simultaneously deplete iron levels in cells, i.e. have a ‘specific’ and a ‘non-specific’ component to their mode of action. Here, as a proof of principle, we report the successful design of such dual-action inhibitors based on the analysis of small molecule iron complexes along with their synthesis, structure-activity analyses and cell-based evaluation. ‘Non-denaturing’ electrospray ionization mass spectrometry (ESI-MS) was used as a principal analytical tool in identifying inhibitors leading to the desired metal binding stoichiometry.

Results and Discussion

The objective of our study was to design PHD2 inhibitors that bind ferrous iron at its active site and deplete cellular iron levels by inducing the binding of a second iron ion. We began by the analysis of reported crystal structures of small molecule-iron complexes²⁷ and more complex supramolecular structures.²⁸ These structures revealed that diacylhydrazines can form octahedral complexes with iron characterized by a tridentate chelation mode²⁷ (Fig. 1D and E). Notably, these structures demonstrate that a single diacylhydrazine moiety has the potential to simultaneously chelate a second transition metal ion²⁸ when appropriately functionalized with additional metal-chelating groups (e.g. phenolic hydroxyl groups or heteroaromatic sp² nitrogens) as shown in Fig. 1F and G. We thus envisaged that, in contrast to the bidentate chelation mode observed with all previously reported PHD2 inhibitors, diacylhydrazines may also enable the binding of a second transition metal ion at the active site in an analogous manner to the iron chelation observed with functionalized diacylhydrazines in the small-molecule crystal structures (Fig. 1F and G). We conceived that the chelation of a ‘first’ transition metal by the possibly deprotonated diacylhydrazine motif might structurally pre-organize the inhibitor to support chelation of a second metal ion.

Initial docking studies using the GOLD software^{29, 30} suggested that the diacylhydrazine **1** (Fig. 2) may bind at the PHD2.Fe active site. Therefore, compound **1** was prepared and tested for binding to the catalytic domain of PHD2 (residues 181–426, hereafter PHD2) using the ESI-MS (which has previously been found to be useful for assaying ligand binding to the catalytic domain of PHD2^{31–33} and other 2OG oxygenases).^{24, 34–36}

ESI-MS studies

ESI-MS studies on PHD2 in the presence of two equivalents of ferrous sulfate implied that hydrazide **1** forms a PHD2.Fe₂.**1** complex as the major observed new species under standard assay conditions (Fig. 3A). The PHD2.Fe₂.**1** complex peak (27990 Da, peak D, Fig. 3A) has a molecular mass of 290 Da larger than that of the no inhibitor control (PHD2.Fe, 27700 Da, peak B, Fig. 3A), corresponding to the mass of diacylhydrazide **1** (237 Da) and a second iron (56 Da). Note that there is a small peak (27755 Da, peak C, Fig. 3A) corresponding to a PHD2.Fe₂ complex in the no inhibitor control which may result from non-specific binding of Fe(II) ions to the PHD2 protein under the standard assay conditions. In contrast, the 2OG co-substrate or 2OG-competitive inhibitors such as NOG or BIQ do not induce binding of a second iron to PHD2 (Supplementary Fig. 1).³⁷

To investigate the role of iron in the binding of **1** to PHD2, we conducted binding experiments in the presence of apo-PHD2 (PHD2 without the ferrous iron) and two

equivalents of Zn(II), Cd(II), Ni(II) or Mn(II), respectively. Notably, only Zn(II) gave rise to small peaks that may correspond to PHD2·Zn·**1** and PHD2·Zn₂·**1** complexes (Supplementary Fig. 2). These results imply that under standard conditions, ferrous ions, but not any other tested transition metal ions (except possibly Zn(II)), are required for the formation of di-metal complexes between diacylhydrazine **1** and PHD2.

To explore the molecular features that are required for the binding of the second iron ion, analogs of diacylhydrazine **1** (Fig. 2) were then synthesized. Variants of the sidechain (**2-5**) and the pyridine ring (**6-10**) of hydrazine **1** were prepared. Compounds **1-10** were obtained from monoacylhydrazines (Scheme 1) either *via* ring opening of anhydrides (**Method A**), 1-hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) coupling with the monomethyl adipate followed by basic hydrolysis (**Method B**), or by nucleophilic substitution with ethyl pentafluorophenyl fumarate followed by basic hydrolysis (**Method C**).

ESI-MS studies imply that the binding affinities of diacylhydrazines gradually decrease with increasing size of the side chains (Fig. 3). Glutarate derivative **2** formed a two-iron complex with PHD2 (peak E, Fig. 3A); however, no detectable complex was observed with adipate derivative **3**, possibly due to the extended aliphatic side chain of **3** which might hinder efficient binding of the compound into the PHD2 2OG binding pocket. Interestingly, the maleate derivative **4** was able to induce binding of a second iron to PHD2 (peak G, Fig. 3A), whereas the isomeric fumarate derivative **5** only bound to one iron (peak H, Fig. 3B), revealing that the stereochemistry of the olefinic side chain affects the binding of the second iron ion. When the 2-pyridyl group of the parent compound **1** was replaced by a phenyl (**6**), 3-pyridyl (**8**), or 4-pyridyl (**10**) group, the analogues lost their ability to chelate a second iron (Fig. 3B), suggesting a specific regiochemical requirement of the pyridyl-*N* to bind a second iron.

We considered that the introduction of an electron-donating dimethylamino group to the *para*-position of aromatic ring of **1** (compound **7**) might increase the binding affinity for a second iron. However, this substitution led to a decreased formation of the two iron binding complex (peak K, Fig. 3B) and an increased formation of a single iron complex (peak J, Fig. 3B). We hypothesized that this may be due to the stronger iron chelating ability of **7** which may result in the binding of **7** to free Fe(II) in the solution, therefore limiting the amount of Fe(II) available for two iron binding complex formation. This is supported by subsequent ESI-MS experiments (Fig. 4) with varying concentrations of Fe(II) and **7**. The results imply that the single iron binding complex (PHD2·Fe·**7**, peak C, Fig. 4) is predominantly formed when Fe(II) is limiting, whereas two iron binding complex (PHD2·Fe₂·**7**, peak D, Fig. 4) is predominantly observed when Fe(II) is present in excess.

In general, appropriately functionalized bicyclic inhibitors have been reported to be more potent against PHD2 than their monocyclic analogs (provided they fit into the enzyme active site), in part likely because the second ring of the inhibitors can form hydrophobic interactions with the amino acid residues located at the entrance of the PHD2 active site (Fig. 1B).³⁸ We therefore prepared bicyclic (**11-24**, Fig. 2) and tricyclic (**25-28**, Fig. 2) analogs of **1** using similar methods to those described above. Consistent with the ESI-MS

results of the monocyclic derivatives, most of the bicyclic and tricyclic compounds (**11-28**) are able to induce the binding of a second iron to PHD2 (Table 1). Substantial single iron complex peaks were also detected in some of the two iron binding compounds under standard conditions (*i.e.* succinates **15**, **25** and maleate **27**), however, when Fe(II) is present in excess, only two iron binding complex peaks were observed (data not shown). As anticipated, 3-quinolinyl derivatives (**21-23**) (scaffold **G**, Fig. 2) and fumarate derivatives (**24**, **28**) only bind to PHD2 with a single iron (Table 1). These results show that the ability of a diacylhydrazine derivative to induce a second iron binding to PHD2 is strongly affected by its side chain and the orientation of the aromatic ring.

Further studies were carried out to compare the PHD2 binding affinities of selected diacylhydrazide derivatives with those of the unreactive 2OG analogue NOG and BIQ (Fig. 1). The ESI-MS competition experiments using equimolar of NOG or BIQ revealed that they could substantially compete with the diacylhydrazides for binding to the PHD2 active site (Supplementary Fig. 1). These results therefore indicate that the diacylhydrazides bind specifically to the 2OG binding site in PHD2 as reported for NOG³⁷, BIQ³⁷ and related compounds.³⁸ The single iron binding 2-pyridine derivatives (**8**, **9**) and 3-quinoline derivatives (**21**, **24**), as well as the two iron binding 3-isoquinoline **11** are among the few diacylhydrazines that were shown to compete effectively with NOG for binding to PHD2 under standard conditions (Supplementary Fig. 1).

Docking studies

We have been unable to obtain crystal structure of PHD2 complex with a diacylhydrazine, so to investigate the potential binding modes of the diacylhydrazines, we performed docking studies (Supplementary Information) with selected compounds using the GOLD software.^{29, 30} A proposed binding mode for the diacylhydrazines that induce binding of a second iron ion is shown in Fig. 5A. Compounds that bind PHD2 (based on ESI-MS), such as the succinate (**1**), glutarate (**2**, not shown), maleate (**4**) and fumarate (**5**) derivatives, gave rise to at least one predicted docking mode displaying bidentate coordination of the catalytic Fe(II) atom with concomitant salt bridge formation between Arg383 of PHD2 and the carboxylate group of a ligand (Fig. 5). In contrast, adipate **3**, which did not bind to PHD2, failed to dock into the binding site.

For compounds capable of a second iron binding, at least one binding mode with three hydrogen bond acceptor atoms (typically an sp²-hybridized nitrogen atom, a diacylhydrazine oxygen atom and diacylhydrazine nitrogen atom) located on one side of the ligand is apparently required, as illustrated for the maleate derivative **4** (Fig. 5C). Inhibitor binding to the active site iron could cause structural pre-organization of these acceptor atoms to support binding of the second iron. Interestingly, the *trans*-geometry of the sidechain in the fumarate derivative **5** resulted in docking poses with the amide carbonyl of the fumarate sidechain pointing towards the entrance of the active site (Fig. 5D), thus ablating the alignment of three acceptor atoms which we propose to be required for second iron binding. This is consistent with the ESI-MS data demonstrating that none of the fumarate derivatives **5**, **9**, **14** and **28** were able to induce the binding of a second iron binding (Table 1).

To validate the docking data and to investigate residues that may contribute to binding of the second iron ion, we performed site-directed mutagenesis on PHD2. Residues that could potentially be involved in the induction of the second iron based on crystal structures of PHD2-inhibitor complexes (PDB IDs: 2HBT, 2G1M³⁹, 3HQR³⁷ were identified and replaced with non-chelating analogs. In particular, the active site variants Y303A, Y310F, D254A, M299V and Y329F were prepared and analyzed for binding with **1** by ESI-MS (Fig. 3C). All tested variants had some affinity for the binding of **1** with two iron ions, supporting the proposal that chelation of the second iron results from the diacylhydrazines. However, the Y310F, and in particular Y303A variant resulted in a substantial reduction in the binding affinity of **1**. This observation supports a potential role for Y303 in binding of the second iron ion, in accordance with the binding mode proposed in Fig. 5A, perhaps *via* coordination of its phenolic oxygen. In the case of Y310, it is unlikely that the phenolic oxygen directly coordinates to the 'second' iron due to its position in the PHD2 active site.

Inhibitory potencies of the diacylhydrazines

In vitro PHD2 inhibition studies were then carried out using an amplified luminescent proximity homogeneous assay (ALPHA), hereafter referred to as the AlphaScreen assay⁴⁰ (Table 1). Initial two-concentration screening at 10 μ M and 300 μ M of compounds revealed that the two iron binding monocyclic diacylhydrazines **1**, **2** and **4** and bicyclic diacylhydrazines **15–20** are weak PHD2 inhibitors. These results are in broad concordance with the ESI-MS competition studies, which indicate their weak binding to PHD2 in the presence of equimolar of the unreactive 2OG analog NOG. Subsequent investigation to determine the half maximal inhibitory concentration (IC₅₀) of the active compounds revealed that while all the single iron binding diacylhydrazides including the fumarate derivatives [**5** (IC₅₀ 47 μ M), **9** (IC₅₀ 0.082 μ M), **14** (IC₅₀ 9.7 μ M), and **28** (IC₅₀ 54 μ M)], the 3-pyridine **8** (IC₅₀ 40 μ M), and the 3-quinoline **21** (IC₅₀ 21 μ M) display the typical sigmoidal inhibition curves, the active two-iron binding compounds exhibit an atypical bimodal type of inhibition curves (Fig. 6). Some compounds interfere with the AlphaScreen assay, particularly when used at high concentrations; however, controls revealed that this does not appear to be the case for these two-iron binding compounds (data not shown). The fact that the bimodal curve was only observed with the active two-iron binding compounds led to the hypothesis that this could result from two (or more) general mechanisms of PHD2 inhibition by these compounds: one via binding to the enzyme active site and another via chelation of free Fe(II). We propose that the bimodal inhibition curve is a result of the changing relative concentrations of equilibrating apo-PHD2, PHD2.Fe, PHD2.Fe.inhibitor, PHD2.Fe₂.inhibitor and inhibitor_n.Fe complexes. The ESI-MS data for **7** provide some support for this proposal (Fig. 4). When Fe(II) is present in excess of the compound **7** (Fig. 4C), the inactive PHD2.Fe₂.**7** complex is observed but as the Fe(II) concentration is lowered in comparison to the compound concentration (Fig. 4B), the active PHD2.Fe complex is observed, in concordance with the apparently reduced inhibition observed in the AlphaScreen assay at higher compound concentrations (phase 2 of the bimodal curve, Fig. 6). However, when the compound is present in excess over Fe(II) (Fig. 4A), the amount of inactive apo-PHD2 increases with increasing **7** (possibly consistent with phase 3 of inhibition curve observed in the AlphaScreen assay, Fig. 6), most likely due to the chelation of free Fe(II) in solution by **7**. The operation of the two inhibition mechanisms results in the

difficulty in determining the IC_{50} values for the active two-iron binding compounds. To allow a rough estimation of inhibitory potencies, we calculated the IC_{50} s by analyzing the first phase (sigmoidal) of the inhibition curve [for compounds **7** (IC_{50} 0.3 μ M) and **25** (IC_{50} 0.4 μ M)], or by removing the two outlying points [for compounds **11** (IC_{50} 18.4 μ M) and **12** (IC_{50} 24.6 μ M)], as indicated in Fig. 6.

Overall, our ESI-MS binding studies indicate that the PHD2 binding affinities of the diacylhydrazines and their abilities to induce the binding of a second iron to the enzyme active sites may, at least to an extent, reflect their inhibitory potencies (*i.e.* a stronger ligand is generally expected to be a more potent inhibitor as demonstrated by compounds **8**, **9**, **11**, and **21**); although it is not always the case. For instance, compound **24** (a single-iron binding compound) which substantially competes with NOG in the ESI-MS studies only showed weak inhibitory potency ($IC_{50} > 300 \mu$ M). The differences observed in the binding affinities and inhibitory potencies of the inhibitors may reflect the different assay conditions used in the ESI-MS (*i.e.* gas phase) and AlphaScreen (*i.e.* aqueous phase) experiments.

Cell-based studies

We proceeded to investigate the cell-based activity of selected diacylhydrazide compounds, using HIF-1 α stabilization as an indicator for cellular PHD2 inhibition (Fig. 7). Initially, we considered the possibility that at least some of the compounds might be cell-permeable in their free acid form due to their structural similarity to the reported cell-based HIF prolyl hydroxylase inhibitor BIQ (Fig. 1C).⁴¹ Diacylhydrazine derivatives bearing a free carboxylate group with *in vitro* PHD2 inhibitory activity (compounds **7**, **11** and **12**, Table 1) did not up-regulate HIF-1 α levels in human hepatoma Hep3B cells (data not shown), suggesting that the diacylhydrazine or carboxylic acid moieties negatively affect cell-penetrating ability. Therefore, for the subsequent cell-based testings, we used ester derivatives, which in general are more cell-penetrating than their respective free acids.⁴² The requisite methyl or ethyl esters (**29-44**) were obtained from the corresponding acylhydrazines by acylation (Scheme 1).

Hep3B cells were exposed to the esters at two concentrations (0.1 mM and 1 mM) under normoxic conditions for 6h. The cell-based HIF hydroxylase inhibitor dimethyl oxalylglycine^{16, 43} (**DMOG**, Fig. 1C) was used as a positive control and DMSO as a negative control. Consistent with *in vitro* inhibition data of their corresponding acids (*i.e.* **7**, **11**, and **12**) we observed a clear induction of HIF-1 α levels by compounds **30** (data not shown), **32**, and **33** (Fig. 7A and B) in a concentration-dependent manner. Compounds **35-38** were also shown to effectively upregulate HIF-1 α at higher concentration (1 mM), despite the high IC_{50} values for their corresponding acids (*i.e.* **15**, **16**, **18**, **19**) $> 300 \mu$ M. Notwithstanding with its relatively strong binding affinity and inhibition potency of its corresponding acid **21** (IC_{50} 21 μ M), the ester **39** failed to induce HIF-1 α accumulation (data not shown). Compounds **42** and **43** were not tested in cell-based assays due to their low solubility in DMSO (Table 1).

For selected compounds, we then investigated the contribution of iron chelating ability to their cellular 2OG oxygenase inhibitory potency in Hep3B cells. For this purpose, cells were

incubated with the iron chelator DFO or DMOG (as positive controls) as well as compound **30**, in the absence or presence of equimolar Fe(II), which were added either at the beginning or during the final 2 h of a 6 h incubation period. As expected, DFO, DMOG and **30** induced HIF-1 α stabilization (Fig. 7B); however, in the presence of an equimolar amount of Fe(II), HIF-1 α stabilization was not observed with **DFO** or **30** (Fig. 7B). Titrations using different Fe(II) concentrations demonstrated that induction of HIF-1 α by **30** was reduced as the iron concentration was increased (Fig. 7C). These observations suggest that, at least in part, the cellular activity of **30** may be due to iron chelation. Interestingly, however, we observed that addition of an equimolar amount of Fe(II) with DMOG also resulted in a reduction in HIF-1 α stabilization, compared to DMOG alone. NOG and DMOG are relatively poor iron chelators, hence these results suggest a more complex relationship between iron availability and the HIF system than currently understood. We also cannot rule out the possibility that the addition of iron may shift the equilibrium between uncomplexed compound and (charged) compound-Fe(II) complexes, thus altering the amount of compound that is available for cellular uptake.^{27, 44}

We then tested diacylhydrazine derivatives containing the fumarate sidechains (**29**, **31**, **34**, **41** and **44**) in Hep3B cells. In contrast to non-olefinic derivatives which stabilized HIF-1 α in a concentration-dependent manner (Fig. 7A), the fumarate derivatives, **29** and **34** displayed better HIF-1 α stabilization at lower concentration (i.e. HIF-1 α stabilization at 0.1 mM compound >> HIF-1 α stabilization at 1 mM compound) in Hep3B cells (Fig. 7D and E), possibly due to the interaction of these compounds with factors working to destabilize HIF-1 α at higher concentration. Notably, **34** gave rise to detectable HIF-1 α induction at concentrations as low as 25 μ M, and HIF-1 α levels obtained at 50 μ M of **34** were in fact greater than those obtained with the established cell-active HIF hydroxylase inhibitor DMOG at 100 μ M. On the other hand, **29** showed similar level of HIF-1 α stabilization with DMOG at 100 μ M. The ester form of **9** (IC₅₀ 0.082 μ M) (**31**) was only slightly active in the tested cells, possibly due to the low cell-permeability of the monocyclic compound. Compound **44** has low solubility in DMSO and was therefore not tested in cells.

Notably, in RCC4/VHL cells (RCC4 cells are renal carcinoma cells lacking VHL; RCC4/VHL cells are RCC4 cells that have been engineered to produce VHL), some compounds including **30** displayed better HIF-1 α upregulation at higher (1mM) concentration, while others including **29** and **34** gave rise to higher HIF-1 α levels when used at the lower concentration (200 μ M) (consistent with the observation in Hep3B cells) (Fig. 7F). In the RCC4/VHL cell line, **29** displayed better HIF-1 α upregulation than **34**; in contrast in Hep3B cells where these compounds displayed similar potency, suggesting the cell-type dependent effect of these compounds. The free acid of **29** (**5**) at 1mM, also upregulated HIF-1 α , but to a lesser extent than its ester, likely due to reduced cell permeability. Similarly, the free acid of **34** (**14**) showed almost no HIF-1 α induction (Fig. 7F). To test whether the observed HIF-1 α upregulation in cells is due to the inhibition of the HIF prolyl and asparaginyl hydroxylases, we utilized hydroxyl-residue-specific HIF-1 α antibodies.⁴¹ Experiments were performed in VHL-defective renal carcinoma cells carrying an empty vector (RCC4) using compounds **29** and **30**, along with **DMOG** as positive control (Fig. 7G). In RCC4 cells, the VHL-mediated HIF-1 α degradation pathway is blocked,

allowing simultaneous measurement of inhibition of HIF prolyl- and asparaginyl-hydroxylase activity in cells. In contrast to DMOG which clearly shows a preference for inhibition of FIH over the PHDs, **29** inhibits HIF prolyl-hydroxylation (Hyp564) more than asparaginyl-hydroxylation (HyAsn803). Similarly, **30** showed partial selectivity for HIF prolyl-hydroxylation over asparaginyl-hydroxylation.

Interestingly, despite the similar level of HIF-1 α induction by DMOG (1mM), **29** (0.2 mM) and **30** (1 mM) (Fig. 7F) in RCC4/VHL cells, these compounds showed different levels of inhibition on HIF prolyl-hydroxylation in RCC4 cells (Fig. 7G). HIF prolyl-hydroxylation was only partially inhibited by DMOG and **30** at high concentrations (1 mM and 2 mM), compared to **29**, which almost completely inhibit HIF prolyl-hydroxylation at concentration as low as 0.2 mM. We therefore speculate that the upregulation of HIF-1 α by DMOG and **30** may not be solely due to the inhibition of HIF prolyl-hydroxylation.

Conclusions

The results demonstrate that appropriately substituted aryl-diacylhydrazines can act as HIF hydroxylase inhibitors, as indicated by results with isolated PHD2 and by the application of modification specific antibodies in cells. The ESI-MS experiments with recombinant PHD2 imply that some, but not all, of the diacylhydrazine-based inhibitors can induce binding of a second iron ion at the active site of PHD2. Whether or not binding of a second ion occurs is dependent both on the substitution pattern on the aromatic ring and the side chain structure.

In terms of their mode of action the diacylhydrazine PHD-inhibitors are related to a family of serine protease inhibitors that bind to their target enzymes and achieve selectivity by inducing the binding of a zinc ion that is not normally present at the active site.⁴⁵ However, in the case of the diacylhydrazine-based HIF hydroxylase inhibitors, depletion of cellular iron has functional consequences because non-specific iron chelators can induce HIF-1 α and thereby other HIF target genes, including two of the three PHD isoforms (PHD2 and PHD3).^{1, 3-5} Thus, the diacylhydrazine compounds have the potential to act as dual-action inhibitors in cells, by directly inhibiting the HIF hydroxylases (and at least with the current compounds, likely inhibiting other 2OG oxygenases as well) and generally depleting iron by enabling its sequestration at the active sites of the PHDs and other 2OG oxygenases. Further structure-activity studies should enable the balance between these inhibitor modes to be altered, e.g. by increasing binding to specific 2OG oxygenases. Although there may be concerns as to the stability of acylhydrazines *in vivo* with associated safety issues, we note that monoacylhydrazines derivatives have found applications as peptide deformylase inhibitors,^{46, 47} and diacylhydrazines have been extensively used as insecticides because of their structural similarities to the insect molting hormone 20-hydroxyecdysone.⁴⁸⁻⁵⁰ Although we are aware that some diacylhydrazines may have toxicity issues in mammalian cells, the work on insecticides⁴⁸ suggests that modification of the substitution patterns may give rise to compounds with acceptable safety profiles and without significant toxicity to mammals.

Finally, non-heme iron-dependent oxygenases can be categorized into those that employ one or two active site ions. The identification of compounds that induce binding of a second ion

to a mono-iron oxygenase provides a link between the two subfamilies, and may help to enable protein-engineering studies aimed at the development of new catalytic activities.

Experimental

Non-denaturing ESI-MS experiments

The catalytic domain of PHD2 (residues 181-426) was desalted using a Bio-Spin 6 Column (Bio-Rad, Hemel Hempstead, UK) into 15 mM ammonium acetate (pH 7.5).^{31, 32} This stock solution was diluted with the same buffer to a final concentration of 100 μ M. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in 20 mM HCl to a concentration of 100 mM. This was then diluted with deionized water to give a final working concentration of 100 μ M. The protein was mixed with Fe(II) and a potential inhibitor to give final concentrations of 15 μ M PHD2, 30 μ M Fe(II), and 15 μ M inhibitor. In the ESI-MS studies with varying Fe(II) and inhibitor concentrations, concentrations of these reagents were adjusted according to *n* equivalent of PHD2 concentration. The solution was then incubated for 10 minutes at room temperature prior to ESI-MS analysis. Data were acquired on a Q-TOF mass spectrometer (Q-TOF micro, Micromass, Altrincham, UK) interfaced with a NanoMate device (Advion Biosciences, Ithaca, NY, USA) with a chip voltage of 1.70 kV and a delivery pressure 0.5 psi (1 psi = 6.81 kPa). The sample cone voltage was typically 80 V with a source temperature of 40 °C and with an acquisition/scan time of 10s/1s. Calibration and sample acquisition were performed in the positive ion mode in the range of 500-5,000 *m/z*. The pressure at the interface between the atmospheric source and the high vacuum region was fixed at 6.60 mbar. External instrument calibration was achieved using sodium iodide. Data were processed with MASSLYNX 4.0 (Waters).

AlphaScreen PHD2 inhibition assay

Full method for PHD2 inhibition assay will be reported elsewhere. Standard reaction consisted of PHD2 (1 nM), Fe(II) (20 μ M), ascorbate (200 μ M), 2OG (2 μ M), biotinylated 19-mer CODD peptide (DLDLE MLAPYIPMDDDFQL) (60 nM) and inhibitor (varies) in 2% DMSO, 50mM HEPES, 0.01% Tween-20, 0.1% BSA. Details of the *N*-terminally truncated PHD2 was prepared as reported.^{39, 51} The IC₅₀ values were calculated using nonlinear regression with normalized dose-response fit on Prism GraphPad.

Cell-based experiments

Effects of potential inhibitors on HIF-1 α levels were assessed in human Hep-3B hepatoma cells, and RCC4/VHL renal carcinoma cells re-expressing VHL.⁵² To compare the effect of diacylhydrazine derivatives on HIF-1 α prolyl and asparaginyl hydroxylation, VHL-defective renal carcinoma cells (RCC4/VA) incapable of proteasomal HIF-1 α degradation were used. Confluent cells were incubated for 5 h or 6 h in medium supplemented with inhibitor (dissolved in DMSO, stock concentration of 100 mM). DMSO was used as negative control, and DMOG and BIQ were used as positive controls. Experimental details of HIF stabilization assay and HIF hydroxylase assay have been reported.^{41, 53} In summary, immunoblots of the whole cell lysates were probed with HIF-1 α antibody or HIF-1 α hydroxyl-residue specific antibodies; hydroxy-Pro564 (Hyp564) and hydroxy-Asn803 (HyAsn803). HIF-1 α band intensities were used to normalize hydroxylation signals in

RCC4/VA cells. Primary antibodies used were, mouse anti- β -tubulin (Sigma), β -actin/HRP (Abcam), mouse anti-HIF-1 α (BD Transduction Laboratories, clone 54), rabbit anti-Hyp564 (Millipore Biosciences), and mouse anti-HyAsn803 was a gift from Dr M. K. Lee.⁵⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2OG	2-oxoglutarate
HIF	hypoxia inducible factor
PHD	prolyl hydroxylases
CODD	C-terminal oxygen-dependent degradation domain
Hyp564	hydroxy-Pro564
CAD	C-terminal transactivation domain
HyAsn803	hydroxy-Asn803
NOG	N-oxalylglycine
DMOG	dimethyloxalylglycine
ESI-MS	electrospray ionization mass spectrometry
Hep	human hepatoma cells
RCC	renal carcinoma cells

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Fig. 1.

Structural considerations for the design of diacylhydrazines as ‘dual-function’ inhibitors of the HIF prolyl hydroxylases. (A) The reaction catalyzed by the HIF prolyl hydroxylases (PHD enzymes). (B) View from a crystal structure of PHD2 in complex with a bidentate iron chelating inhibitor **5**.²⁶ (C) Structures of inhibitors of 2OG dependent oxygenases that act *via* generic iron chelation, such as deferoxamine **2**, or by competition with the 2OG co-substrate, such as *N*-oxalyl amino acids including *N*-oxalyl glycine **3**,³⁷ and isoquinoline compound **5**.²⁶ Dimethyloxalyl glycine (DMOG) **4** is a cell-penetrating form of **3**. Ligand atoms involved in Fe(II) chelation are shown in bold. (D-G) Views derived from representative small-molecule crystal structures of diacylhydrazine-iron complexes.^{27, 55} (D, E) Views derived from a crystal structure of a diacylhydrazine-iron complex with tridentate iron chelation (CSD ID OKUGIK²⁷). (F, G) View derived from a crystal structure of a diacylhydrazine-iron complex with concomitant bidentate and tridentate iron chelation (CSD ID QEVBEY⁵⁵).

Fig. 2.

Scaffolds used for the development of diacylhydrazine derivatives as PHD2 inhibitors. Cell-based activity assays were carried out for selected diacylhydrazines (in their methyl or ethyl ester forms, compounds highlighted in blue). *In vitro* potency and cell-based activity data are summarized in Table 1. Some compounds were not tested, including those with limited solubility or due to interference with the biochemical assays.

Fig. 3. Mass-spectrometric analysis of the monocyclic diacylhydrazines that apparently induce binding of a second iron ion to PHD2. (**A, B**) Deconvoluted ESI-MS spectra under non-denaturing conditions for in the presence of 2 equiv. of Fe(II) ions and 1 equiv. of monocyclic diacylhydrazine derivatives **1-10**. There is a small peak (peak C) in the control (without inhibitors) corresponding to a PHD2.Fe₂ complex which may result from non-specific binding of Fe(II) ions to the PHD2 under the standard assay conditions. (**C**) Deconvoluted ESI-MS spectra for five active site variants of PHD2 in the presence of 2

equiv. of ferrous ions and 1 equiv. of compound **1**; (a) Y303A, (b) Y310F, (c) D254A, (d) M299V and (e) Y329F PHD2. PHD2 variants were purified as *N*-terminally His6-tagged proteins; removal of the His6-tag with thrombin leaves six additional residues (GSHMAS) at the *N*-terminus meaning the variants have higher molecular masses than the untagged proteins used in (**A**, **B**).

Fig. 4.

Non-denaturing ESI-MS studies demonstrate differential binding modes of **7** under different Fe(II) concentrations. (A) When **7** is in excess (5 equiv.) and Fe(II) is limiting (0.5 equiv.), an increase of apo-PHD2 (peak A) and PHD2.Fe.**7** (peak C) is observed, demonstrating the ability of the **7** to chelate free Fe(II) in the solution. (B) When Fe(II) is equimolar to compound **7** (1 equiv.), **7** binds to PHD2 with either single Fe (peak C) and dual Fe (peak D) (top spectra). (C) When Fe(II) is in excess (5 equiv.), **7** binds to PHD2 with dual Fe (peak D, top).

Fig. 5. Possible structural explanation for diacylhydrazine-induced binding of a second iron at the PHD2 active site. (A) Proposed ligand interaction diagram for diacylhydrazines that induce binding of a second iron in the PHD2 active site. The model for this potential binding mode was derived from structure-activity data (Table 1) followed by docking analyses. (B, C and D): Representative binding modes for inhibitors **1**, **4** and **5** obtained from docking analyses using the GOLD software. Selected residues of the PHD2 active site and the active site catalytic iron atom (red) are highlighted. For docking, protein coordinates were derived from the co-crystal structure of PHD2 with the bicyclic isoquinoline inhibitor **BIQ** (PDB ID 2HBT). Proposed locations for binding of the second iron (green) are indicated. Note that the experimental results reveal that the succinate-(**1**) and maleate-(**4**) derivatives bind with two irons, but the fumarate-(**5**) derivative with only one iron.

Fig. 6.

Compounds that bind to single Fe display typical inhibition curve, whereas dual Fe binding compounds display atypical bimodal type of inhibition curve. Results are from AlphaScreen, $n = 3$. Points removed for rough estimation of IC_{50} values are highlighted in dashed boxes. The three phases of the bimodal inhibition curve are indicated by black bars (phase 1, 2 and 3).

Fig. 7.

Effects of diacylhydrazine-based compounds on cellular HIF-1α levels. (A, D) Immunoblots demonstrating HIF-1α stabilisation in the presence of diacylhydrazine derivatives (1 mM and 0.1 mM) in Hep3B cells after 6 h incubation. (B, C) Effects of the addition of Fe(II) ions on the HIF-1α induction caused by **30** after 6 h incubation. No Fe(II) indicates no addition of extra Fe(II). (E) Dose-dependent HIF-1α induction caused by **29** and **34** after 6 h incubation. (F) Concentration-dependent stabilisation of HIF-1α in RCC4/VHL cells by diacylhydrazine derivatives **5**, **14**, **29**, **30** and **34**. (G) Effects of **29** and **30** on HIF prolyl and

asparaginyl hydroxylation in VHL-defective RCC4 cells after 5 h incubation (HIF-1 α band intensities were used to normalize hydroxylation signals, tubulin/ β -actin protein levels were used as loading controls). DMSO (1%) was used as a negative control; dimethyloxalylglycine (DMOG) and bicyclic isoquinoline inhibitor (BIQ) were used as positive controls.

Scheme 1.

Synthetic procedures for preparation of diacylhydrazine derivatives as potential PHD2 inhibitors. **Method A**: carboxylic acid anhydride, EtOAc. **Method B**: monomethyl adipate, 1-hydroxybenzotriazole (HOBt), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), Et₃N, THF, then NaOH, THF. **Method C**: i) ethyl pentafluorophenylfumarate, THF:EtOAc 1:1; ii) LiOH, THF:H₂O 1:2. See Fig. 2 for full structures of individual compounds.

Table 1Summary of the *in vitro* and cell-based potencies of the diacylhydrazines as PHD inhibitors.

Cmpd.	Scaffold ^a	Core	Sidechain	PHD2 IC ₅₀ ^b	Cell-based activity ^c HIF-1α Induction	PHD2·Fe _n ·Cm pd. Complexes in ESI- MS ^d	
						n=1	n=2
	N-oxalyl glycine, NOG (DMOG)			10μM	Active	+	
	2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido)acetic acid, BIQ			0.3μM	Active	+	
1	A	2-Pyridyl	Succinate	>300μM	–		+
2	A	2-Pyridyl	Glutarate	>300μM	–		+
3	A	2-Pyridyl	Adipate	>300μM	–	no binding	
4	A	2-Pyridyl	Maleate	>300μM	–		+
5 (29)	A	2-Pyridyl	Fumarate	47μM	Active	+	
6	B	Phenyl	Succinate	>300μM	–	+	
7 (30)	B	4-Dimethyl-	Succinate	0.3μM [*]	Active		+
8	C	3-Pyridyl	Succinate	40μM	–	+	
9 (31)	C	3-Pyridyl	Fumarate	0.082μM	Active	+	
10	C	4-Pyridyl	Succinate	>300μM	–	+	
11 (32)	D	3-Isoquinoliny	Succinate	18.4μM [*]	Active		+
12 (33)	D	3-Isoquinoliny	Glutarate	24.6μM [*]	Active		+
13	D	3-Isoquinoliny	Maleate	>300μM	–		+
14 (34)	D	3-Isoquinoliny	Fumarate	9.7μM	Active	+	
15 (35)	E	2-Quinoliny	Succinate	>300μM	Active		+
16 (36)	E	2-Quinoliny	Glutarate	>300μM	Active		+
17	E	2-Quinoliny	Maleate	>300μM	–		+
18 (37)	F	1-Isoquinoliny	Succinate	>300μM	Active		+
19 (38)	F	1-Isoquinoliny	Glutarate	>300μM	Active		+
20	F	1-Isoquinoliny	Maleate	>300μM	–		+
21 (39)	G	3-Quinoliny	Succinate	21μM	Inactive	+	
22 (40)	G	3-Quinoliny	Glutarate	>300μM	Inactive	+	
23	G	3-Quinoliny	Maleate	>300μM	–	+	
24 (41)	G	3-Quinoliny	Fumarate	>300μM	Inactive	+	
25 (42)	H	3-9H-Pyrido	Succinate	0.4μM [*]	ND		+
26 (43)	H	3-9H-Pyrido	Glutarate	0.13uM [*]	ND		+
27	H	3-9H-Pyrido [3,4- <i>b</i>]indolyl	Maleate	ND	–		+
28 (44)	H	3-9H-Pyrido	Fumarate	54μM	ND	+	

^a See Fig. 2 for structures of inhibitor scaffolds.^b Determined by AlphaScreen assay in the presence of 1 nM recombinant PHD2 enzyme and 2 μM of 2OG co-substrate. IC₅₀ values were determined by fitting the dose-response curve as described in the methods section.

^c The corresponding methyl or ethyl esters (compound numbers in parentheses) of the diacylhydrazines were tested in the cell-based assays. ND, not determined (including compounds that were not well soluble, or interfered with the assay).

^d Number of iron ions bound in the most abundant PHD2-Fe_n-compound complex in nondenaturing protein ESI-MS experiments under standard conditions (n=1, 2).

* Compounds that display bimodal inhibition curve. IC₅₀ values were determined as described in the main text.