Selective Small Molecule Probes for the Hypoxia Inducible Factor (HIF) Prolyl Hydroxylases

Rasheduzzaman Chowdhury,[†] José Ignacio Candela-Lena,[†,‡,§] Mun Chiang Chan,[†,§] David Jeremy Greenald,[∥,⊥] Kar Kheng Yeoh,[†,#] Ya-Min Tian,[¶] Michael A. McDonough,[†] Anthony Tumber,[▲] Nathan R. Rose,[†] Ana Conejo-Garcia,[†] Marina Demetriades,[†] Sinnakaruppan Mathavan,[∥] Akane Kawamura,[†] Myung Kyu Lee,[○] Freek van Eeden,[⊥] Christopher W. Pugh,[¶] Peter J. Ratcliffe,[¶] and Christopher J. Schofield[†,*]

[†]Department of Chemistry, Chemistry Research Laboratory, University of Oxford, Mansfield Road, Oxford, OX1 3TA, United Kingdom

[‡]Departmento de Química Orgánica y Farmacéutica, Facultad de Farmacia, Universidad de Sevilla, E-41071 Sevilla, Spain

[∥]Department of Stem Cell and Developmental Biology, Genome Institute of Singapore, A*STAR, Singapore, Singapore

[⊥]Department of Biomedical Science, University of Sheffield, Western Bank, Sheffield S10 2TN, United Kingdom

[¶]Nuffield Department of Clinical Medicine, Henry Wellcome Building for Molecular Physiology, University of Oxford, Roosevelt Drive, Oxford OX3 7BN, United Kingdom

[▲]Structural Genomics Consortium, University of Oxford, Headington, Oxford OX3 7DQ, United Kingdom

[○]BioNanotechnology Research Center, KRIBB, 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea

Ⓢ *Supporting Information*

ABSTRACT: The hypoxia inducible factor (HIF) system is central to the signaling of low oxygen (hypoxia) in animals. The levels of HIF-α isoforms are regulated in an oxygen-dependent manner by the activity of the HIF prolyl-hydroxylases (PHD or EGLN enzymes), which are Fe(II) and 2-oxoglutarate (2OG) dependent oxygenases. Here, we describe biochemical, crystallographic, cellular profiling, and animal studies on PHD inhibitors including selectivity studies using a representative set of human 2OG oxygenases. We identify suitable probe compounds for use in studies on the functional effects of PHD inhibition in cells and in animals.
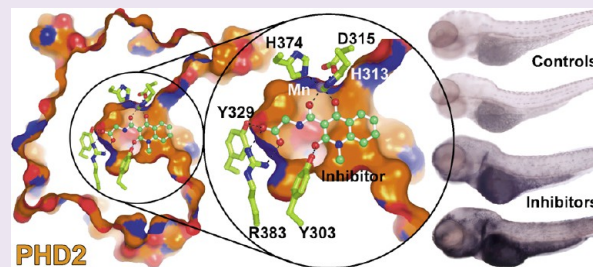
Hypoxia is linked to human diseases including anemia, cancer, and cardiovascular, pulmonary and neurodegenerative disorders. In animals, the response to hypoxia is mediated by upregulation of the α,β-heterodimeric hypoxia inducible transcription factor (HIF). HIF coordinates the regulation of gene arrays that work to ameliorate the effects of hypoxia in a context-dependent manner (for reviews, see refs 1−3). Under normoxic conditions, HIF-α degradation is signaled for by the catalytic action of the HIF-prolyl hydroxylases (PHD or EGLN 1−3 isoforms), which are 2-oxoglutarate (2OG) dependent oxygenases (Figure 1). Hydroxylation of HIF-α at either or both of two prolyl-residues signals for its ubiquitination and proteosomal degradation by enhancing binding to the von Hippel Lindau protein (VHL), which is a targeting component of a ubiquitin ligase. The most important of the human PHDs is PHD2.[4] In an additional mechanism, present in all higher animals but only in some lower animals,[5] the *C*-terminal transcriptional activation domain (CAD) of HIF-α undergoes asparaginyl hydroxylation catalyzed by Factor Inhibiting HIF (FIH), which reduces the interaction of HIF with transcriptional coactivators (CBP/p300). The requirement of PHD and FIH for

oxygen coupled to appropriate kinetic and biological properties is proposed to enable them to act as hypoxia "sensors" for the HIF system.

Inhibition of the PHDs is being pursued as a therapy for conditions including anemia and ischemic/vascular diseases.[6−8] The HIF hydroxylases are part of the wider family of >60 human 2OG oxygenases (for reviews see 9,10). 2OG oxygenases have roles in collagen and carnitine biosynthesis, lipid metabolism, histone/nucleic acid modification/repair, ribosome hydroxylation and modification of epidermal growth factor-like domains, and RNA splicing related proteins.[9−13] Following from the demonstration that nonspecific inhibitors can induce the HIF-mediated hypoxic response,[14,15] various PHD and FIH inhibitors were reported in the academic and patent literature.[6−8,16,17] However, in few cases has the selectivity of HIF hydroxylase inhibitors with respect to other 2OG oxygenases involved in
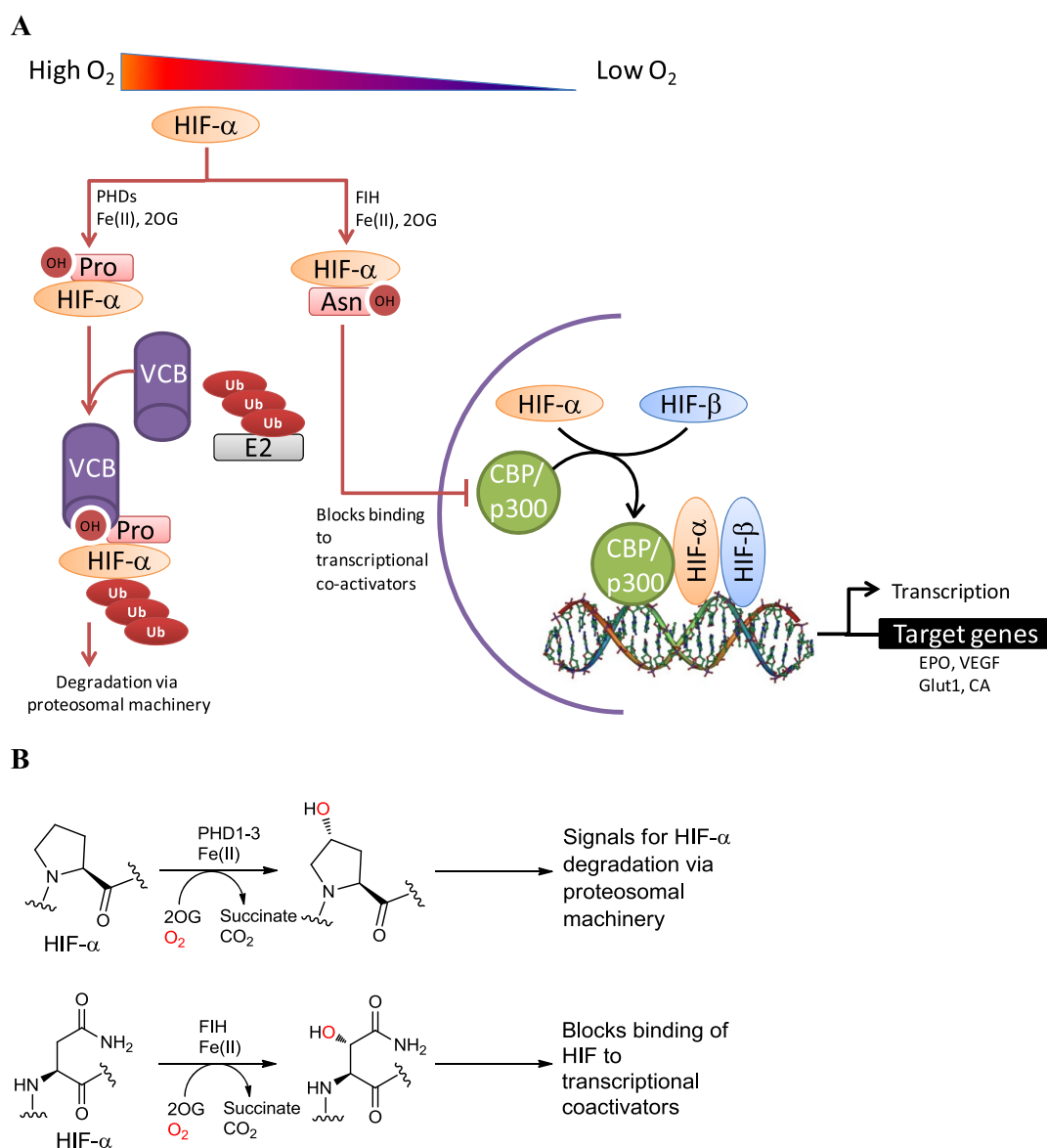
Figure 1. Roles of the 2OG dependent hydroxylases in the regulation of hypoxia inducible factor (HIF). (a) Human HIF oxygen-sensing mechanism highlighting the roles of HIF hydroxylases as oxygen sensors. The catalytic activity of the PHDs and FIH signals for degradation *via* the proteosomal machinery or reduces binding of the CBP/p300 transcriptional coactivator proteins, respectively. HIF-α prolyl-hydroxylation increases binding to the von Hippel-Lindau (VHL) protein which is part of a complex with elongins B and C (VCB) that triggers E2 ligase mediated ubiquitination of HIF-α. (b) Stoichiometry of 2OG oxygenase catalyzed HIF hydroxylations. Ub = ubiquitin, CBP = CREB (cAMP-response element-binding) binding protein, EPO = erythropoietin, VEGF = vascular endothelial growth factor, Glut1 = Glucose transporter 1, CA = carbonic anhydrase.

regulation, been reported.[18] Emerging roles for 2OG oxygenases, in particular in the regulation of gene expression *via* histone/nucleic acid demethylation and hydroxylation, raises questions as to the selectivity of the reported HIF hydroxylase inhibitors, and the extent to which their biological activity is mediated solely by inhibition of the PHDs and FIH. Given that PHD inhibitors are currently in late stage clinical trials, the issue of selectivity is important. Here we describe the identification of potent and selective PHD inhibitors that will be of use to the academic and pharmaceutical communities as probes for studying the biological roles of the PHDs and for their validation as clinically viable targets.

■ RESULTS AND DISCUSSION

With the aim of identifying selective PHD inhibitors, we established fluorescence- and luminescence-based *in vitro* assays

for representative human 2OG oxygenases. These comprised assays for PHD2, and representative members of different 2OG-dependent histone demethylases (JMJD2A, JMJD2C, JMJD2E, FBXL11, JMJD3, JMJD1A and JARID1C) that employed Amplified Luminescent Proximity Homogeneous Assay (AlphaScreen) methods.[19,20] A peptide corresponding to the HIF-1α C-terminal oxygen-dependent degradation domain (CODD, residues 556−574) was used as a substrate in the PHD2 assays with an antibody selective for the prolyl-hydroxylated form of CODD being used to detect the reaction product *via* AlphaScreen methodology (Supplementary Figure 1, Supporting Information).

PHD2 and FIH are inhibited by simple acyclic/cyclic 2OG analogs, including *N*-oxalylglycine (NOG/ **1a**) and pyridine-2,4-dicarboxylate (**2a**).[14,15,21−24] The dimethyl ester form of NOG (DMOG, **1b**) has been widely used as a hypoxia mimic in studies
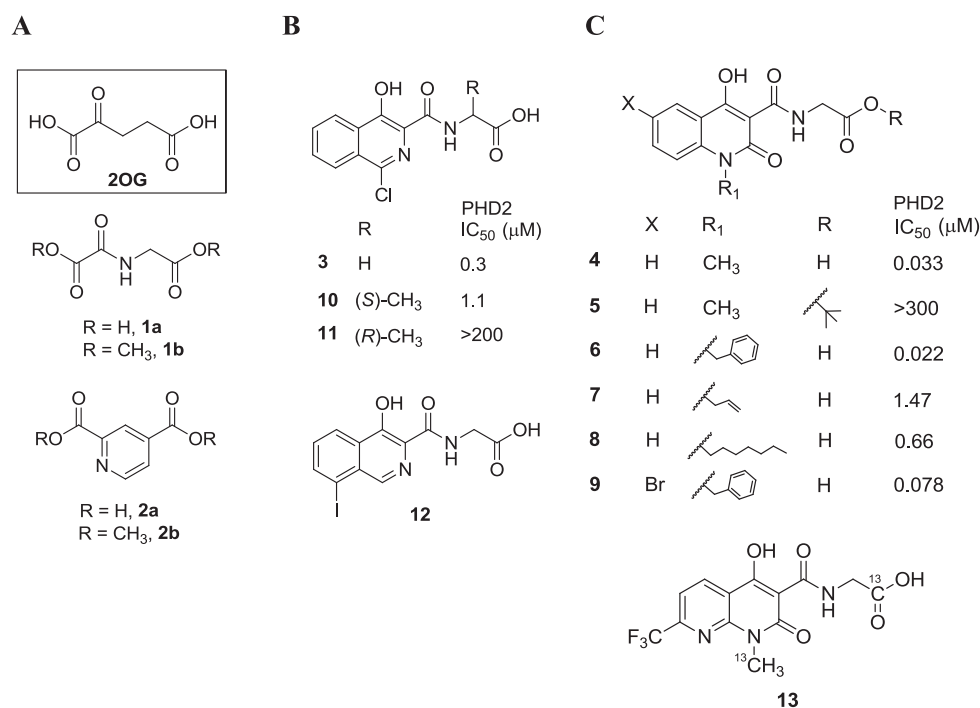
Figure 2. Identification of selective PHD2 inhibitors. Assay results for (a) relatively simple 2OG analogs and (b, c) more potent and selective heterocyclic compounds.

Table 1. Inhibition Potencies of 4HQ Compounds against a Panel of Histone Demethylases[a]

	JMJD2A	JMJD2C	JMJD2E	JMJD3	JMJD1A	FBXL11	JARID1C
3	>20	>20	>20	>20	>20	>20	549.6
4	>100	>100	>100	>100	>100	100	443.2
6	100	100	>100	>100	>100	52	159
7	>100	>100	>100	>100	>100	>100	465
8	43	86	99	>100	>100	>100	122.9
9	(100)[b]	93	(81)[b]	>100	>100	>100	>100

[a]IC$_{50}$s are in μM. Substrates used in the assays for Jumonji C (JmjC)-domain containing proteins, JMJD2A, JMJD2C, JMJD2E, JMJD3, JMJD1A, FBXL11 (F-box and leucine-rich repeat protein 11) and JARID1C (Jumonji/ARID domain-containing protein 1C) were histone H3 fragment substrates Biotin-H3(7-14)K9me3 (for JMJD2 subfamily), Biotin-H3(14-34)K27Me3, H3(1-21)K9Me2-Biotin, Biotin-H3K36(28-48)Me2 and H3(1-21)K4Me3-Biotin, respectively. [b]Values in parentheses are for % inhibition at 100 μM.

employing cells. Although the extent to which these compounds inhibit different 2OG oxygenases varies, most simple 2OG analogs inhibit a substantial set of human 2OG oxygenases;[19,25] hence, their value as probes for dissecting the functions of individual (or subfamilies of) oxygenases is limited. The results of screening against our panel of 2OG oxygenases support the proposal that **1a** and **2a** are insufficiently selective in their inhibition to be classified as selective probes for studying the role of PHDs in the hypoxic response.[19] In order to identify a suitable PHD selective inhibitor for use as a functional probe, we evaluated a set of heterocyclic compounds, some of which have been reported as PHD inhibitors in the academic and patent literature.[6,8]

Compound **3** (IOX3) has been developed as a PHD inhibitor to upregulate endogenous EPO (PHD2 IC$_{50}$ = 1.4 μM using a fluorescence-based assay[8,26]); it inhibits *via* competition with 2OG and bidentate iron-chelation (see below). 4-Hydroxy-2-oxo-1,2-dihydroquinoline glycinamide analogs (4HQs), including compound **4**, are structurally related PHD inhibitors (PHD2 IC$_{50}$ = 0.31 μM for **4**[8,27]), which may have a potentially different metal coordination mode. We synthesized 4HQ derivatives (**4–9**) and isoquinolines (**3, 10, 11**) and tested their potencies

against PHD2 using the AlphaScreen methodology (Figure 2, inhibition plots are in Supplementary Figure 2, Supporting Information). The results reveal that some of the 4HQ derivatives are potent PHD2 inhibitors (IC$_{50}$ < 1 μM), with **4** and **6** being the most potent of the compounds tested (IC$_{50}$ = 0.033 μM and 0.022 μM, respectively). This is in accord with the reported IC$_{50}$ values for 4HQs ranging from 0.001 to 1 μM and EC$_{50}$ (half maximal effective concentration) values ranging from 1 to 20 μM for EPO.[8,27] Differences in our IC$_{50}$ values and those reported likely reflect variations in assay conditions, in particular the low concentration of PHD2 used in the AlphaScreen assay. These compounds display better PHD2 inhibition potencies than the isoquinoline derivatives **3, 10** and **11** (IC$_{50}$ ≥ 0.3 μM) (Figure 2) and the generic 2OG oxygenase inhibitor, NOG/**1a** (IC$_{50}$ ≈ 10 μM) (Figure 2). The reduced activity of the isoquinolines with L(*S*)-, compared to the D(*R*)-alanyl side chain (in **10** and **11**, respectively) can be rationalized by better fit of the former in the 2OG binding pocket of PHD2.[21,28] The ester **5** was not a PHD inhibitor, likely because esterification reduces binding in the 2OG binding pocket, which involves ionic interactions.[28]

To investigate whether the inhibitors selectively inhibit PHD2, we then tested them against a panel of other human 2OG
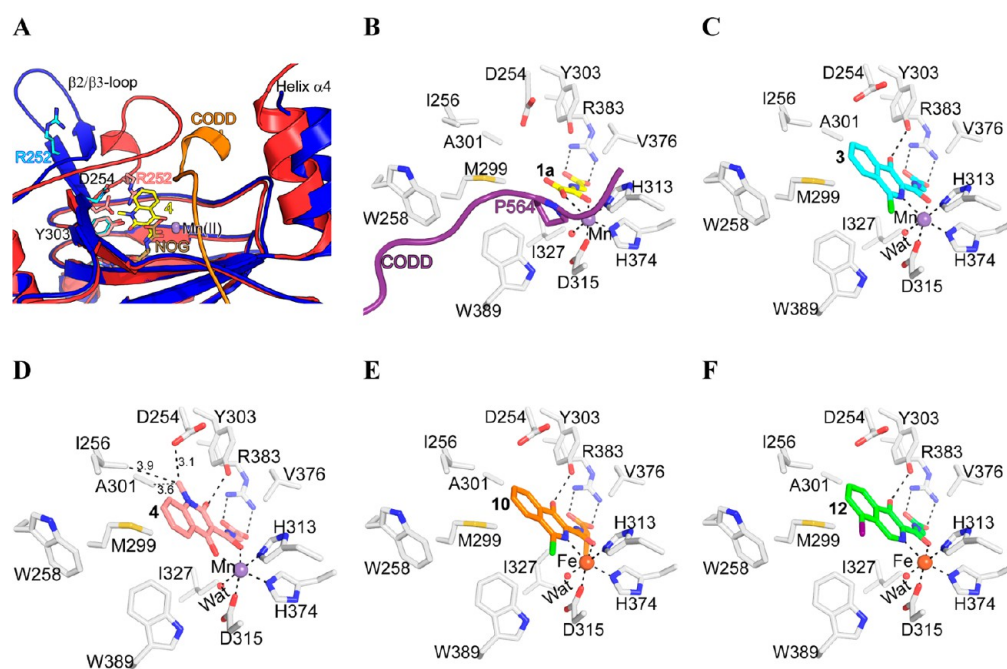
Figure 3. Views from structures of PHD2 in complex with inhibitors. (a) Superimposition of PHD2.Mn(II).4 (blue ribbons, PDB ID: 4BQW) and PHD2.Mn(II).1a.CODD (red ribbons, PDB ID: 3HQR) structures showing how **4** (and most bicyclic inhibitors) may block productive binding of the conserved LXXLAP motif of the substrate. Compound **4** inhibits PHD2 competitively with respect to 2OG; the amide carbonyl of **4** coordinates the iron *trans* to the Asp315 Oδ1 and its phenolic-O *trans* to His374 Nε2. Views from PHD2 structures in complex with (b) **1a** and CODD (purple, PDB ID: 3HQR)[21], (c) **3** (PDB ID: 4BQX), (d) **4** (PDB ID: 4BQW), (e) **10** (PDB ID: 4BQY) and (f) **12** (PDB ID: 2G19)[28].

oxygenases focusing on histone N^ε-methyl lysine demethylases (KDMs). The results indicate that **4** and **6** are at least 2−5000 fold selective, as judged by IC_{50} values, for PHD2 over the KDMs (Table 1) and FIH (data not shown). These results indicate that the 4HQ compounds are good candidates for use as functional probes for PHD activity.

To investigate the mechanism of PHD inhibition, we carried out crystallographic analyses on **3**, **4** and **10** using the PHD2 catalytic domain (residues 181−426). The overall folds in the structures for complexes with **3**, **4** and **10** are similar to that observed for PHD2 in complex with **12** (PDB ID: 2G19)[28] with an RMSD range of ≤0.4 Å for the Cα atoms of residues 188−402. There are differences between the isoquinoline (**3**, **10** and **12**) and 4HQ (**4**) compound classes with respect to metal coordination and active site binding. Analyses of the structures reveal that the isoquinolines (**3**, **10** and **12**) bind the PHD2 active site metal in a near identical manner (Figure 3).[28] In the following paragraphs, we mainly discuss the complex with **12**, as representative of the isoquinolines, in comparison with the structure of a PHD2.Mn(II).4 complex (Figure 3).

In the PHD2.Mn(II).4 structure, **4** is positioned similar to **12** in complex with PHD2.Fe,[28] that is, both inhibitors "slot" into the relatively narrow obloid-shaped active site opening. The bicyclic heteroaromatic rings of **4** and **12** are sandwiched between the hydrophobic side chains of Tyr310, Met299 and Trp389.[28] Like 2OG[16]/NOG[21] and **12**, **4** coordinates Mn(II) (substituting for Fe(II)) in a bidentate fashion (Figure 3a,d); similarly, the side chain carboxylate of **4** is positioned to hydrogen bond with Arg383 and Tyr329 (Arg383 NH1-O3, 2.8 Å; Arg383 NH2-O1, 2.9 Å; and Tyr329 OH-O1, 2.6 Å) (Figure 3d). Both **4** and **12** use their side chain amide-carbonyl to chelate the metal. However, unlike **12** which employs its aromatic nitrogen as a second ligand, **4** uses its phenolic oxygen as the second ligand to form a six-membered chelate ring (NHCO-

Mn(II); 2.1 Å; O4−Mn(II), 2.0 Å). The phenol of Tyr303 is positioned to hydrogen bond to quinoline-2-OH (Tyr303 OH-O, 2.7 Å) of **4** or to the analogously positioned isoquinoline-4-OH of **12**. In the PHD2.Fe(II).10 structure, the methyl group of the L(*S*)-Ala side chain interacts with residues Ile327 (βIII) and Leu343 (βII) (which form part of the double-stranded-β-helix (DSBH) core fold of PHD2/2OG oxygenases). In contrast, the methyl of the D(*R*)-Ala side chain of **11** would likely cause steric hindrance with the side chains of Val376 (DSBH βVII), consistent with an approximately 200-fold lower IC_{50} for **10** (1.1 μM) compared to **11** (>200 μM) (Figure 2).

Comparison of the PHD2.4/12 structures with that of PHD2 in complex with its HIF-1α C-terminal oxygen-dependent degradation domain (CODD) substrate (HIF-1α residues 556−574) and NOG (PDB ID: 3HQR)[21] shows binding of **4**/**12** likely blocks productive binding of the conserved LXXLAP motif of HIF-1α. The aromatic rings of **4**/**12** project through the active site opening and likely make a steric clash with the hydroxylated HIF-1α residue Pro564 (Figure 3).

While crystallographic analyses imply that **4** (predominantly) binds PHD2 in one particular orientation (Figure 3 and Supplementary Figure 3, Supporting Information), an NMR study suggests that a close analog of **4** (**13**, Figure 2) binds to the metal in the active site *via* two different modes in solution: "X", as observed in the PHD2.Mn(II).4 crystal structure and an "F", flipped conformation.[29] In the "F" orientation, the quinoline ring is flipped by rotating 180° (relative to "X") around the bond between the quinoline ring and the side chain so that the quinoline-2-OH of **13** coordinates the metal (Supplementary Figure 3b, Supporting Information). While the bicyclic quinoline ring maintains hydrophobic contacts in both orientations, the *N*-methyl substituent at the quinoline nitrogen fits well only in the "X" orientation, where it binds in a shallow cleft formed by the Asp254, Ile256 and Ala301 side chains (Figure 3d). Modeling
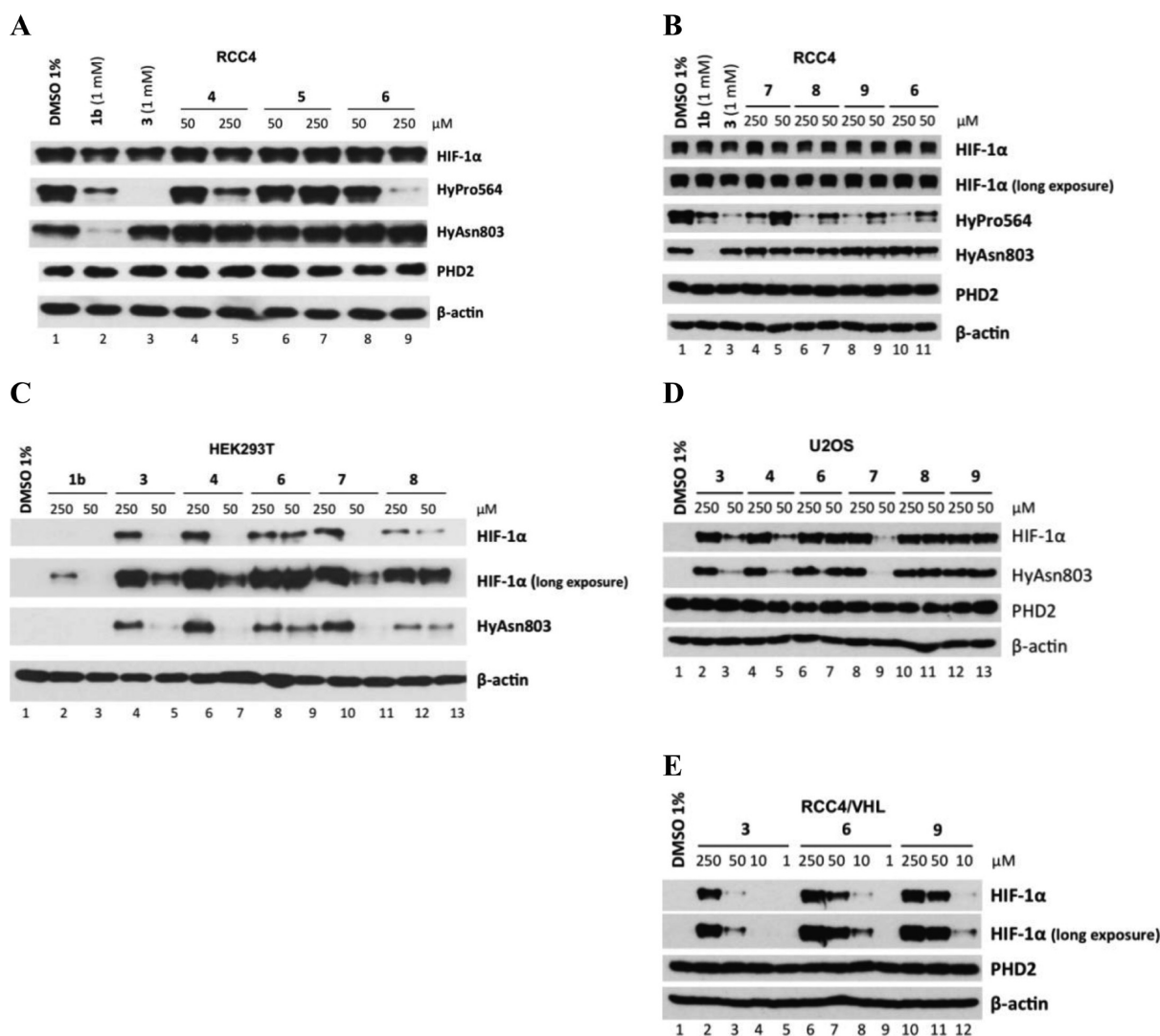
1491

dx.doi.org/10.1021/cb400088q | *ACS Chem. Biol.* 2013, 8, 1488−1496

Figure 4. Selective inhibition of the PHDs in human cell lines. (a-b) Selectivity of the tested inhibitors for HIF-1α prolyl- over asparaginyl- hydroxylation in RCC4 after 6h of treatment. Upregulation of HIF-1α by inhibitors in various cell types (after 6h of treatment): HEK293T (c), U2OS (d) and RCC4 stably-transfected with *C*-HA-tagged wildtype VHL (e). See text for assay descriptions.

studies predict that compounds with bulkier groups (as in **6−9**) at the quinoline nitrogen will make steric clashes with this hydrophobic cleft and will likely adopt the "F" orientation (Supplementary Figures 3 and 4, Supporting Information). This prediction is consistent with the structure−activity relationship (SAR) studies of 4HQ derivatives with various alkyl/aryl substitutions at the quinoline nitrogen which reveal that the *N*-benzyl derivative (**6**) is a potent inhibitor (Figure 2).

We then evaluated the inhibitors in cells. We first tested the efficacy of the selected PHD inhibitors in a human renal carcinoma (RCC4) cell line lacking VHL, which is required for the degradation of hydroxylated HIF-α (Figure 4a,b). We used the antibodies selective for hydroxylated form(s) of HIF-1α[24] to probe the compounds for inhibition of HIF prolyl- (PHD-catalyzed) and asparaginyl-(FIH-catalyzed) hydroxylation. This enables direct comparison of effects on Pro- and Asn-hydroxylation without confounding from preferential degradation of prolyl hydroxylated HIF-1α. The results were compared to those with the nonselective inhibitor, DMOG (**1b**) (Figure 4). Consistent with the *in vitro* data, all of the active 4HQ derivatives inhibit CODD hydroxylation (Figure 4a,b). Inhibition of CAD hydroxylation was not observed at the concentrations tested.

Ester **5** (IC$_{50}$ > 300 μM) did not inhibit HIF hydroxylation (Figure 4a), consistent with the isolated protein results.

The active 4HQ compounds (**3, 4, 6−9**) were further tested in VHL-competent cell lines, including human embryonic kidney (293T), human bone osteosarcoma (U2OS) and RCC4 re-expressing VHL (RCC4/VHL) cells (Figure 4c−e). Increased HIF-1α levels were used as an indicator for PHD inhibition. The 4HQ compounds (**3, 4, 6−9**) effectively increase HIF-1α levels in all tested VHL-competent cell lines (Figure 4c−e), showing that their inhibitory effects are independent of cell type. Stabilized HIF-1α was substantially hydroxylated at Asn803 (Figure 4), confirming the selectivity for PHD over FIH inhibition. In comparison to DMOG, HIF-1α induction by the 4HQ derivatives is several fold higher, suggesting that they are more potent PHD inhibitors in cells. Their *in vitro* potencies are generally reflected in their ability to induce HIF, with **6** (IC$_{50}$ = 0.022 μM) being the most effective followed by **4** (IC$_{50}$ = 0.033 μM) and **9** (IC$_{50}$ = 0.078 μM). Dose response experiments in RCC4/VHL cells reveal that **6** and **9** increase HIF-1α to a higher level than with the same concentrations of **3** (Figure 4e). Taken together, the results reveal that the 4HQ compounds **4**, and **6−9** are potent and selective cellular PHD inhibitors.
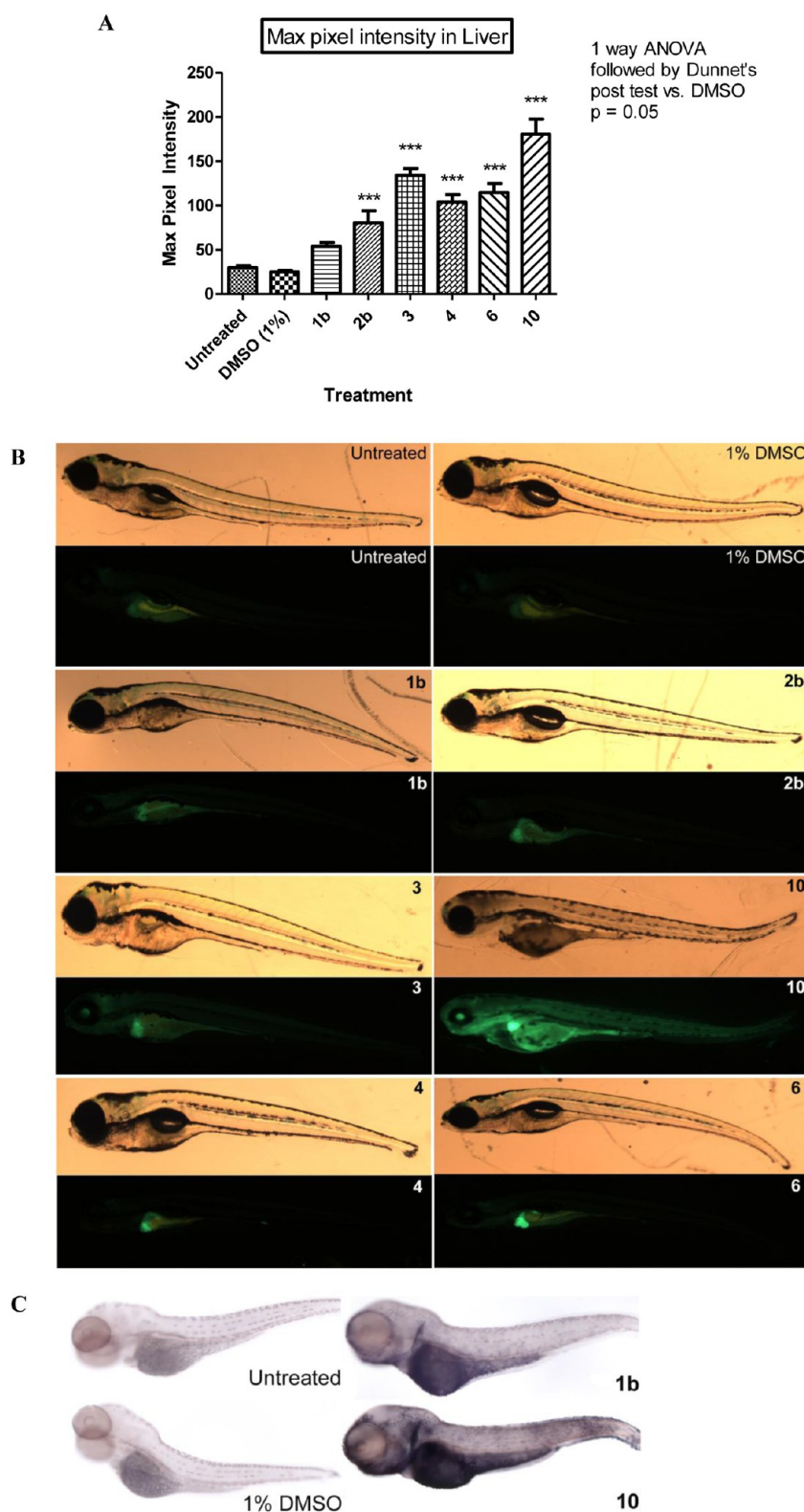
Figure 5. Inhibition of the PHDs in Zebrafish embryos. (a) Maximum pixel intensity in liver of phd3:gfpsh144/sh144 embryos (treated for 48 h); significance calculated using 1 way ANOVA followed by Dunnet's post test, * indicates $p < 0.05$ ($n = 8$). (b) Wild-type larva showing transgene-mediated phd3:GFP fluorescence. (c) Whole mount in situ hybridization of wild-type larva showing increased phd3 expression with inhibitors. DMOG (**1b**) acts as a positive control. Compounds tested are denoted by numbers in the panels.

To investigate the utility of the compounds as *in vivo* functional probes, we tested selected compounds (**1b**, **2b**, **3**, **4**, **6** and **10**) to upregulate HIF signaling in a whole organism, that is, transgenic zebrafish (*Danio rerio*). Because the expression of

the PHD3 encoding gene is regulated by HIF in humans and zebrafish, PHD3 levels are a readout of HIF activity. A zebrafish hypoxia reporter line was generated expressing GFP with the phd3 promoter elements.[30] Transgenic wild-type embryos at 3

days postfertilization treated with compounds (10 μM) for 2 days displayed clear increase in phd3:EGFP expression in the liver, relative to controls (Figure 5a). Significant increases in GFP levels were observed with **2b, 3, 4, 6** and **10** (Figure 5a,b). Transcriptional activation of endogenous phd3 by **10** was demonstrated by whole mount in situ hybridization of 3 days postfertilization embryos treated for 8 h (Figure 5c). In contrast, when in situ hybridization was performed after 48 h treatment, no phd3 expression was discerned for **10** (data not shown), suggesting that the activity of the inhibitor in zebrafish or the compound stability in E3 medium might be time-limited. These data show that the inhibitors can activate an *in vivo* real-time hypoxic reporter in a vertebrate model organism to a greater extent than the widely used nonspecific DMOG (**1b**) and validate their use as *in vivo* functional probes.

Overall, the work has defined a set of 2OG competitors that are potent and selective inhibitors of the PHDs, as isolated proteins, in cells and in an animal model. Several of these compounds are of interest as functional probes. On the basis of the combined results available, we have selected **6** as a preferred functional probe; **6** is commercially available as IOX2.[31] Finally, it is important to point out that although the compounds are active as PHD inhibitors, our selectivity panel is non-comprehensive; there are ~60 human 2OG oxygenases including other prolyl-3- and 4-hydroxylases such as the collagen prolyl hydroxylases. Thus, it cannot be ruled out that some of the biological observations in the case of the compounds involve inhibition by enzymes other than the PHDs.

■ METHODS

Production of Recombinant Proteins. PHD2 (residues 181−426) production used pET28a(+) vector; purification of recombinant PHD2 was carried out as described.[21] Recombinant Jumonji C (JmjC)-domain containing histone demethylases (KDMs), JMJD2A, JMJD2C, JMJD2E, JMJD3, JMJD1A, FBXL11 (F-box and leucine-rich repeat protein 11) and JARID1C (Jumonji/ARID domain-containing protein 1C) were purified as described.[19]

***In vitro* Hydroxylation Assays for PHD2 (AlphaScreen).** Inhibition assays were carried out in 384-well white ProxiPlates (PerkinElmer) in 10 μL of reaction volume. Standard reaction mixtures consisted of the compound (in 2% DMSO final concentration), enzyme mix (0.001 μM of PHD2, 10 μM of Fe(II), 100 μM of ascorbate) and peptide mix (0.06 μM of biotinylated *C*-terminal oxygen dependent degradation domain (CODD) peptide, 2 μM of 2OG) in 50 mM HEPES pH 7.5, 0.01% Tween-20 and 0.1% BSA buffer. Compounds were preincubated with the enzyme mix for 15 min before being incubated with peptide mix for 10 min at 22 °C. Each reaction was quenched with 5 μL of 30 mM EDTA. The bead mix containing AlphaScreen beads (AlphaScreen streptavidin-conjugated donor and ProteinA-conjugated acceptor beads; PerkinElmer) was preincubated for 1h with a rabbit monoclonal antibody selective for hydroxy-HIF1α (Pro564) (Cell Signaling, Danvers, MA; 1:8000) and were added to the wells for a further 1 h at 22 °C. The plates were then analyzed with an Envision (PerkinElmer) plate reader. The IC_{50} values were calculated using nonlinear regression with normalized dose−response fit using Prism GraphPad ($n \geq 3$).

***In vitro* Demethylation Assays for KDMs (AlphaScreen).** These assays were carried out as described previously.[19]

Cell-based Assays. Experiments were conducted as described.[24] Both VHL-defective (renal carcinoma cells with an empty vector, RCC4) and VHL-competent cells human embryonic kidney HEK293T, osteosarcoma U2OS and RCC4/VHLHA (RCC4 stably transfected with *C*-terminal HA-tagged wt VHL) were used. Cells were treated with DMSO (control) and tested compounds (dissolved in DMSO except for DMOG (**1b**) which was dissolved in PBS and added directly to culture medium) for 4−5 h. Cell extracts were probed with antibodies to

hydroxy-Pro564 (CODD−OH) and hydroxy-Asn803 (CAD−OH). HIF-1α band intensities were used to normalize hydroxylation signals. Antibodies to HIF-1α, CODD−OH and β-actin/HRP were from BD Transduction Laboratories (clone-54), New England Biolabs (clone-D43B5) and Abcam (clone, AC15) respectively and an antibody to CAD−OH was generated as described.[32]

Experiments on Zebrafish. Full details of conditions for the zebrafish assays are reported elsewhere.[30] Phd3:gfp[sh144/sh144] fish (*Danio rerio*) were incrossed to produce phd3:gfp[sh144/sh144] embryos, these were raised at 28 °C in E3 medium. The phd3:gfp[sh144/sh144] line is a hypoxia reporter line created by BAC recombination of the phd3 reporter GFP construct.[30] Adult fishes were maintained on a 14:10-h light/dark cycle at 28 °C in UK Home Office approved facilities in the Medical Research Council Centre for Developmental and Biomedical Genetics aquaria at the University of Sheffield. At 3 days post fertilization, potential inhibitors were added in fresh medium at 10 μM in 1% DMSO. The embryos were incubated with the compounds for a further 48 h. Embryos were anesthetized at 5 dpf by immersion in tricaine (Sigma-Aldrich). Lateral view images of the embryos were taken using a fluorescent dissecting stereomicroscope (Leica) (both bright-field and fluorescent). Fluorescent images were analyzed using Image J.

Whole Mount *in situ* Hybridization. Whole-mount *in situ* hybridization was performed using standard protocols.[33] The phd3 (BC066699) antisense digoxygenin-UTP labeled mRNA probe was synthesized from an expressed sequence tag (EST) clone (RZPD/Imagenes[34]) and the *in situ* images were collected on a Zeiss Axioplan with a 5× objective using a Spot4 digital camera.

Crystallography. PHD2 crystals were grown by vapor diffusion at 20 °C in 300 nL sitting drops with a 1:2 ratio of protein (~1 mM PHD2$_{181−426}$ in 50 mM TRIS-HCl pH 7.5, 2 mM **3**/ **4**/ **10**, 1 mM MnCl$_2$ (when using **3** or **4**)/ 1 mM FeSO$_4$ (when using **10**)) to well solution (100 mM MES-Na pH 6.5, 2 mM MnCl$_2$ /FeSO$_4$, 1.7 M ammonium sulfate and 1% v/v dioxane). Crystals were cryoprotected with 50% Na-malonate pH 6.5 before being cryo-cooled in liquid N$_2$. Data for PHD2 complex with **3**, **4** or **10** were collected from single crystals at 100 K at the Diamond beamline I02 with a Pilatus 6 M detector or using a Rigaku FR-E+ Superbright generator (CuK$_\alpha$ 1.5418 Å) with Saturn 944+ CCD detector or at the European Synchrotron Radiation Facility (ESRF) BM14 beamline with MarCCD detector. The data were processed using Mosflm[35] or HKL2000;[36] the structures were solved by molecular replacement using PHASER[37] (search model PDB ID 2G19). Parameter and topology files for **3**, **4** and **10** were generated using PRODRG.[38] Slowcool-simulated annealing refinement using the maximum-likelihood function and bulk-solvent modeling was carried out using CNS[39] (version 1.3) with iterative rebuilding of the model using COOT.[40] All residues were in allowed regions of a Ramachandran plot as calculated by PROCHECK.[41] Data collection and refinement statistics are in Supplementary Table 1, Supporting Information.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary data. This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: christopher.schofield@chem.ox.ac.uk.

Present Address

#K.K.Y.: School of Chemical Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia

Author Contributions

§J.I.C.-L. and M.C.C. contributed equally to this work

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Chowdhury, R., Hardy, A., and Schofield, C. J. (2008) The human oxygen sensing machinery and its manipulation. *Chem. Soc. Rev. 37*, 1308−1319.

(2) Kaelin, W. G., Jr., and Ratcliffe, P. J. (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol. Cell 30*, 393−402.

(3) Schofield, C. J., and Ratcliffe, P. J. (2004) Oxygen sensing by HIF hydroxylases. *Nat. Rev. Mol. Cell. Biol. 5*, 343−354.

(4) Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., and Pouyssegur, J. (2003) HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. *EMBO J. 22*, 4082−4090.

(5) Loenarz, C., Coleman, M. L., Boleininger, A., Schierwater, B., Holland, P. W., Ratcliffe, P. J., and Schofield, C. J. (2011) The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, Trichoplax adhaerens. *EMBO Rep. 12*, 63−70.

(6) Rose, N. R., McDonough, M. A., King, O. N., Kawamura, A., and Schofield, C. J. (2011) Inhibition of 2-oxoglutarate dependent oxygenases. *Chem. Soc. Rev. 40*, 4364−4397.

(7) Vachal, P., Miao, S., Pierce, J. M., Guiadeen, D., Colandrea, V. J., Wyvratt, M. J., Salowe, S. P., Sonatore, L. M., Milligan, J. A., Hajdu, R., Gollapudi, A., Keohane, C. A., Lingham, R. B., Mandala, S. M., DeMartino, J. A., Tong, X., Wolff, M., Steinhuebel, D., Kieczykowski, G. R., Fleitz, F. J., Chapman, K., Athanasopoulos, J., Adam, G., Akyuz, C. D., Jena, D. K., Lusen, J. W., Meng, J., Stein, B. D., Xia, L., Sherer, E. C., and Hale, J. J. (2012) 1,3,8-Triazaspiro[4.5]decane-2,4-diones as Efficacious Pan-Inhibitors of Hypoxia-Inducible Factor Prolyl Hydroxylase 1−3 (HIF PHD1−3) for the Treatment of Anemia. *J. Med. Chem. 55*, 2945−2959.

(8) Yan, L., Colandrea, V. J., and Hale, J. J. (2010) Prolyl hydroxylase domain-containing protein inhibitors as stabilizers of hypoxia-inducible factor: small molecule-based therapeutics for anemia. *Expert Opin. Ther. Pat. 20*, 1219−1245.

(9) Loenarz, C., and Schofield, C. J. (2008) Expanding chemical biology of 2-oxoglutarate oxygenases. *Nat. Chem. Biol. 4*, 152−156.

(10) Hausinger, R. P. (2004) FeII/alpha-ketoglutarate-dependent hydroxylases and related enzymes. *Crit. Rev. Biochem. Mol. Biol. 39*, 21−68.

(11) Ge, W., Wolf, A., Feng, T., Ho, C. H., Sekirnik, R., Zayer, A., Granatino, N., Cockman, M. E., Loenarz, C., Loik, N. D., Hardy, A. P., Claridge, T. D., Hamed, R. B., Chowdhury, R., Gong, L., Robinson, C. V., Trudgian, D. C., Jiang, M., Mackeen, M. M., McCullagh, J. S., Gordiyenko, Y., Thalhammer, A., Yamamoto, A., Yang, M., Liu-Yi, P., Zhang, Z., Schmidt-Zachmann, M., Kessler, B. M., Ratcliffe, P. J., Preston, G. M., Coleman, M. L., and Schofield, C. J. (2012) Oxygenase-catalyzed ribosome hydroxylation occurs in prokaryotes and humans. *Nat. Chem. Biol. 8*, 960−962.

(12) Klose, R. J., Kallin, E. M., and Zhang, Y. (2006) JmjC-domain-containing proteins and histone demethylation. *Nat. Rev. Genet. 7*, 715−727.

(13) Klose, R. J., and Zhang, Y. (2007) Regulation of histone methylation by demethylimination and demethylation. *Nat. Rev. Mol. Cell. Biol. 8*, 307−318.

(14) Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I.,

Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J., and Ratcliffe, P. J. (2001) *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell 107*, 43−54.

(15) Mole, D. R., Schlemminger, I., McNeill, L. A., Hewitson, K. S., Pugh, C. W., Ratcliffe, P. J., and Schofield, C. J. (2003) 2-oxoglutarate analogue inhibitors of HIF prolyl hydroxylase. *Bioorg. Med. Chem. Lett. 13*, 2677−2680.

(16) Rosen, M. D., Venkatesan, H., Peltier, H. M., Bembenek, S. D., Kanelakis, K. C., Zhao, L. X., Leonard, B. E., Hocutt, F. M., Wu, X., Palomino, H. L., Brondstetter, T. I., Haugh, P. V., Cagnon, L., Yan, W., Liotta, L. A., Young, A., Mirzadegan, T., Shankley, N. P., Barrett, T. D., and Rabinowitz, M. H. (2010) Benzimidazole-2-pyrazole HIF prolyl 4-hydroxylase inhibitors as oral erythropoietin secretagogues. *ACS Med. Chem. Lett. 1*, 526−529.

(17) Tegley, C. M., Viswanadhan, V. N., Biswas, K., Frohn, M. J., Peterkin, T. A., Chang, C., Burli, R. W., Dao, J. H., Veith, H., Rogers, N., Yoder, S. C., Biddlecome, G., Tagari, P., Allen, J. R., and Hungate, R. W. (2008) Discovery of novel hydroxy-thiazoles as HIF-alpha prolyl hydroxylase inhibitors: SAR, synthesis, and modeling evaluation. *Bioorg. Med. Chem. Lett. 18*, 3925−3928.

(18) McDonough, M. A., McNeill, L. A., Tilliet, M., Papamicael, C. A., Chen, Q. Y., Banerji, B., Hewitson, K. S., and Schofield, C. J. (2005) Selective inhibition of factor inhibiting hypoxia-inducible factor. *J. Am. Chem. Soc. 127*, 7680−7681.

(19) Rose, N. R., Woon, E. C., Tumber, A., Walport, L. J., Chowdhury, R., Li, X. S., King, O. N., Lejeune, C., Ng, S. S., Krojer, T., Chan, M. C., Rydzik, A. M., Hopkinson, R. J., Che, K. H., Daniel, M., Strain-Damerell, C., Gileadi, C., Kochan, G., Leung, I. K., Dunford, J., Yeoh, K. K., Ratcliffe, P. J., Burgess-Brown, N., von Delft, F., Muller, S., Marsden, B., Brennan, P. E., McDonough, M. A., Oppermann, U., Klose, R. J., Schofield, C. J., and Kawamura, A. (2012) Plant Growth Regulator Daminozide Is a Selective Inhibitor of Human KDM2/7 Histone Demethylases. *J. Med. Chem. 55*, 6639−6643.

(20) Kawamura, A., Tumber, A., Rose, N. R., King, O. N., Daniel, M., Oppermann, U., Heightman, T. D., and Schofield, C. (2010) Development of homogeneous luminescence assays for histone demethylase catalysis and binding. *Anal. Biochem. 404*, 86−93.

(21) Chowdhury, R., McDonough, M. A., Mecinovic, J., Loenarz, C., Flashman, E., Hewitson, K. S., Domene, C., and Schofield, C. J. (2009) Structural basis for binding of hypoxia-inducible factor to the oxygen-sensing prolyl hydroxylases. *Structure 17*, 981−989.

(22) Hirsila, M., Koivunen, P., Gunzler, V., Kivirikko, K. I., and Myllyharju, J. (2003) Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. *J. Biol. Chem. 278*, 30772−30780.

(23) Koivunen, P., Hirsila, M., Gunzler, V., Kivirikko, K. I., and Myllyharju, J. (2004) Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases. *J. Biol. Chem. 279*, 9899−9904.

(24) Tian, Y. M., Yeoh, K. K., Lee, M. K., Eriksson, T., Kessler, B. M., Kramer, H. B., Edelmann, M. J., Willam, C., Pugh, C. W., Schofield, C. J., and Ratcliffe, P. J. (2011) Differential sensitivity of hypoxia inducible factor hydroxylation sites to hypoxia and hydroxylase inhibitors. *J. Biol. Chem. 286*, 13041−13051.

(25) King, O. N., Li, X. S., Sakurai, M., Kawamura, A., Rose, N. R., Ng, S. S., Quinn, A. M., Rai, G., Mott, B. T., Beswick, P., Klose, R. J., Oppermann, U., Jadhav, A., Heightman, T. D., Maloney, D. J., Schofield, C. J., and Simeonov, A. (2010) Quantitative high-throughput screening identifies 8-hydroxyquinolines as cell-active histone demethylase inhibitors. *PLoS One 5*, e15535.

(26) Warshakoon, N. C., Wu, S., Boyer, A., Kawamoto, R., Sheville, J., Renock, S., Xu, K., Pokross, M., Zhou, S., Winter, C., Walter, R., Mekel, M., and Evdokimov, A. G. (2006) Structure-based design, synthesis, and SAR evaluation of a new series of 8-hydroxyquinolines as HIF-1alpha prolyl hydroxylase inhibitors. *Bioorg. Med. Chem. Lett. 16*, 5517−5522.

(27) Murray, J. K., Balan, C., Allgeier, A. M., Kasparian, A., Viswanadhan, V., Wilde, C., Allen, J. R., Yoder, S. C., Biddlecome, G.,

Hungate, R. W., and Miranda, L. P. (2010) Dipeptidyl-quinolone derivatives inhibit hypoxia inducible factor-1alpha prolyl hydroxylases-1, −2, and −3 with altered selectivity. *J. Comb. Chem. 12*, 676−686.

(28) McDonough, M. A., Li, V., Flashman, E., Chowdhury, R., Mohr, C., Lienard, B. M., Zondlo, J., Oldham, N. J., Clifton, I. J., Lewis, J., McNeill, L. A., Kurzeja, R. J., Hewitson, K. S., Yang, E., Jordan, S., Syed, R. S., and Schofield, C. J. (2006) Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). *Proc. Natl. Acad. Sci. U.S.A. 103*, 9814−9819.

(29) Poppe, L., Tegley, C. M., Li, V., Lewis, J., Zondlo, J., Yang, E., Kurzeja, R. J., and Syed, R. (2009) Different modes of inhibitor binding to prolyl hydroxylase by combined use of X-ray crystallography and NMR spectroscopy of paramagnetic complexes. *J. Am. Chem. Soc. 131*, 16654−16655.

(30) Santhakumar, K., Judson, E. C., Elks, P. M., McKee, S., Elworthy, S., van Rooijen, E., Walmsley, S. S., Renshaw, S. A., Cross, S. S., and van Eeden, F. J. (2012) A zebrafish model to study and therapeutically manipulate hypoxia signaling in tumorigenesis. *Cancer Res. 72*, 4017−4027.

(31) Tocris Biosciences, http://www.tocris.com/; Selleckchem, http://www.selleckchem.com/products/iox2.html; Cayman Laboratories, https://www.caymanchem.com/app/template/Product.vm/catalog/11573.

(32) Lee, S. H., Jeong Hee, M., Eun, Ah. C., Ryu, S. E., and Myung Kyu, L. (2008) Monoclonal antibody-based screening assay for factor inhibiting hypoxia-inducible factor inhibitors. *J. Biomol. Screen. 13*, 494−503.

(33) Thisse, C., and Thisse, B. (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protoc. 3*, 59−69.

(34) van Rooijen, E., Voest, E. E., Logister, I., Korving, J., Schwerte, T., Schulte-Merker, S., Giles, R. H., and van Eeden, F. J. (2009) Zebrafish mutants in the von Hippel-Lindau tumor suppressor display a hypoxic response and recapitulate key aspects of Chuvash polycythemia. *Blood 113*, 6449−6460.

(35) Bailey, S. (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr., D: Biol. Crystallogr. 50*, 760−763.

(36) Otwinowski, Z., and Minor, W. (1997) *Processing of X-ray diffraction data collected in oscillation mode* (Carter, C. W. Jr., and Sweet, R. M., Eds.) in Methods in Enzymology, Vol. 276, pp 307−326, Academic Press, New York.

(37) McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr. 40*, 658−674.

(38) Schuttelkopf, A. W., and van Aalten, D. M. (2004) PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr., D: Biol. Crystallogr. 60*, 1355−1363.

(39) Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr., D: Biol. Crystallogr. 54*, 905−921.

(40) Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr., D: Biol. Crystallogr. 60*, 2126−2132.

(41) Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr. 26*, 283−291.