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A reporter ligand NMR screening method for 2-oxoglutarate oxygenase inhibitors

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Abstract

The human 2-oxoglutarate (2OG) dependent oxygenases belong to a family of structurally related enzymes that play important roles in many biological processes. We report that competition-based NMR methods, using 2OG as a reporter ligand, can be used for quantitative and site-specific screening of ligand binding to 2OG oxygenases. The method was demonstrated using hypoxia inducible factor (HIF) hydroxylases and histone demethylases, and K_D values were determined for inhibitors that compete with 2OG at the metal centre. This technique is also useful as a screening or validation tool for inhibitor discovery, as exemplified by work with protein-directed dynamic combinatorial chemistry (DCC).

Introduction

2-Oxoglutarate (2OG) dependent oxygenases are present in plants, microorganisms and animals.¹⁻⁵ In humans, they are involved in a diverse range of important biological roles, including collagen biosynthesis, oxygen sensing, fatty acid metabolism and epigenetic regulation involving nucleic acid and histone modifications.⁵ Several human 2OG oxygenases, including the hypoxia inducible factor (HIF) hydroxylases,⁶⁻¹⁰ are current inhibition targets for diseases including cancer, ischemia, inflammation and anaemia.¹¹⁻¹⁴ The inhibition of γ -butyrobetaine hydroxylase,¹⁵ a 2OG oxygenase involved in human carnitine biosynthesis, is used clinically for the treatment of angina and myocardial infarction.¹⁶

All 2OG oxygenases utilise 2OG and oxygen as co-substrates to catalyse two-electron oxidation reactions (mostly hydroxylations), with the release of succinate and carbon dioxide as co-products (Supplemental Figure S1). This property has led to the development of several generic activity-based assays (for reviews see references 17 and 18). However, activity-based assays are not always well-suited to the initial stages of medicinal chemistry,

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Supporting Information Available

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e.g. for fragment-based screening, and are only possible when substrates are available. As an alternative to these activity-based assays, binding assays have also been developed for 2OG oxygenases, studying the binding of metal ions, small molecules and other biomolecules. Reported methods include non-denaturing electrospray ionisation mass spectrometry (ESI-MS; for examples see references 19-23), protein X-ray crystallography (for reviews see references 24-26), isothermal titration calorimetry (ITC),²⁷ surface plasmon resonance (SPR),²⁸ circular dichroism (CD),²⁷ electron paramagnetic resonance (EPR)²⁹ and nuclear magnetic resonance (NMR).^{27,30,31} Among these different techniques, non-denaturing ESI-MS has been used by our group as a simple and rapid primary screening method for 2OG oxygenase inhibitors. However, because non-covalent protein-ligand complexes may not always survive the transition from solution phase to gas phase,³² there is a need for complementary solution-based screening techniques.

NMR spectroscopy is an established technique for the study of protein-ligand binding interactions. Ligand detection methods such as saturation transfer difference (STD)³³ and water ligand observed gradient spectroscopy (waterLOGSY)³⁴ are widely used for ligand screening because they do not require isotopically labelled protein and are relatively quick and sensitive. However, many ligand-based NMR methods suffer from limited detection ranges of binding affinities, false positives arising from non-specific binding³⁵ and complications with binding constant determination that arise from, for instance, ligand rebinding events and dependencies on ligand spin relaxation rates and saturation times.^{36,37} NMR reporter screening methods are useful alternatives for the site-specific detection of both high- and low-affinity ligands. By observing changes in the NMR parameter(s) (such as chemical shift or relaxation rate) associated with a reporter ligand, it is possible to obtain qualitative and quantitative information on the binding of ligands that compete with the reporter ligand for the target protein (Figure 1). Several nuclei, including proton,³⁸⁻⁴⁰ phosphorus,⁴¹ fluorine⁴² and carbon⁴³ have been proposed for reporter screening by NMR.

We reasoned that 2OG is a potentially good reporter ligand candidate for generic NMR binding assays for 2OG oxygenases because they all utilise it as co-substrate. Using unlabelled and commercially available [1,2,3,4-¹³C₄]-labelled 2OG ([¹³C]-2OG), we have optimised the experimental conditions and demonstrated the feasibility of applying the reporter ligand method for high-throughput screening and binding constant (K_D) measurements for a selection of 2OG oxygenases.

Results

Methodology

The hypoxia inducible factor (HIF) hydroxylases, in particular the prolyl hydroxylase domain containing enzyme isoform 2 (PHD2; EC 1.14.11.29; for reviews see references 6-8) and factor inhibiting HIF (FIH; EC 1.14.11.30; for reviews see references 9 and 10), play key roles in human oxygen/hypoxia sensing. Their inhibition is of therapeutic interest,¹¹⁻¹⁴ hence they were chosen as model systems.

It is known that the catalytically essential Fe^{II} at the active site of 2OG oxygenases can be substituted by different transition metals.²¹ Because many (but not all) 2OG oxygenases

catalyse 2OG turnover in the absence of substrates, native Fe^{II} was substituted with the diamagnetic Zn^{II} in order to block enzyme-catalysed 2OG turnover and to avoid the oxidation of Fe^{II} to paramagnetic Fe^{III}. In the assays, an eight-fold molar excess of Zn^{II} was used relative to PHD2 to ensure that only the metal-bound *holo* form was present. It should be noted that a caveat of our method is that it does not employ the native metal ion, although the binding affinity of ligands, including 2OG, is not, at least substantially, affected by the use of Zn^{II} as the active site metal (see below; Supplemental Figure S2).

Both PHD2 and FIH form stable complexes with 2OG that saturate at a ~1:1 ratio (Supplemental Figure S3).^{23,31} Under these conditions, the ¹H NMR spectra were dominated by the protein resonances (Supplemental Figure S4). In order to observe the reporter ligand (2OG) signal, the Carr-Purcell-Meiboom-Gill (CPMG)⁴⁴⁻⁴⁶ sequence was applied to attenuate any broad (protein) resonances (Supplemental Figure S4). In particular, the recently proposed Periodic Refocusing Of J Evolution by Coherence Transfer (PROJECT) method,⁴⁷ which uses an additional 90_y° pulse at the midpoint of a double spin echo to refocus and suppress J modulation, provided unperturbed line shapes and was therefore used in assays described herein. This method provides a means to distinguish free from bound 2OG. When 2OG was bound to the protein, significant broadening of the 2OG ¹H resonances was observed due to high ¹H transverse relaxation rate in the bound state, so the resultant CPMG-edited ¹H spectrum displayed near-complete diminution of the 2OG signal intensity. In the presence of a competitive binder, however, 2OG was displaced from the binding site into bulk solution, leading to sharpening of the signals and recovery of the 2OG signal intensity (Figure 2a). The simplicity and sensitivity of the experiment makes it well suited to the primary screening of 2OG oxygenases. In addition, by monitoring the intensity of the 2OG reporter signal as a function of increasing concentrations of an inhibitor (Figure 2b), it is possible to obtain quantitative binding information for the inhibitor (Figure 2c).

When using this method for inhibitor screening, we define the percentage 2OG displacement as:

$$\% \text{ 2OG displacement} = \frac{I_{2\text{OG}} - I_{2\text{OG}(0)}}{I_{2\text{OG}(\text{blank})} - I_{2\text{OG}(0)}} \times 100\% \quad (1)$$

where $I_{2\text{OG}}$ is the integral of the 2OG ¹H signal in the presence of both the inhibitor and the protein, $I_{2\text{OG}(0)}$ is the integral of 2OG in the presence of the protein but in the absence of the inhibitor, and $I_{2\text{OG}(\text{blank})}$ is the integral of 2OG in the absence of both protein and inhibitor. For the ideal scenario in which the 2OG signal is fully attenuated in the presence of the protein (and absence of inhibitor), $I_{2\text{OG}(0)}$ should tend toward zero.

Screening for inhibitor binding

By monitoring the level of 2OG displacement, it is possible to compare the relative strength of binding for different compounds at a single inhibitor concentration in an efficient manner, as was demonstrated herein using several known PHD2/2OG oxygenase inhibitors (Figure 3). Compounds were screened at a concentration of 400 μM in the presence of 10 μM Zn(II)-*holo*-enzyme and 10 μM 2OG.

N-Oxalylglycine (NOG; Figure 3)⁴⁸ is a non-reactive 2OG analogue, with a NH group replacing the CH₂ at the C-3 position of 2OG. It is a relatively non-selective 2OG oxygenase inhibitor,⁴⁹ and gave ~85% 2OG displacement for both PHD2 and FIH (Figure 4). In contrast, the bicyclic isoquinolinyl inhibitor (BIQ; Figure 3)^{50,51} is reported as a potent PHD2 inhibitor. The reporter assay results demonstrate it to be more selective for PHD2 compared with FIH, consistent with literature reports.^{52,53} Full recovery of the 2OG reporter signal was observed with PHD2 but only ~55% 2OG displacement was observed with FIH (Figure 4). The assay was also tested with 5-carboxy-8-hydroxyquinoline (5C8HQ; Figure 3),⁵⁴ a generic inhibitor for histone demethylases,^{55,56} but a weaker inhibitor for HIF hydroxylases.⁵⁴ The single concentration 2OG displacement assay confirms that 5C8HQ binds to both PHD2 and FIH, but its binding is weaker than with NOG (~50% and ~75% 2OG displacement with PHD2 and FIH respectively; Figure 4). Finally, we tested two other known PHD2 inhibitors, including 2,4-pyridine dicarboxylic acid (2,4-PDCA; Figure 3)⁵⁷ and the bicyclic naphthalenylsulfonyl inhibitor (BNS; Figure 3).⁵⁸ Both compounds caused complete 2OG displacement when tested with PHD2 under standard conditions (Figure 4).

Quantification of binding

By monitoring the intensity of the 2OG reporter signal at different inhibitor concentrations, it is possible to obtain the dissociation constant (K_D) for the inhibitor, provided the dissociation constant ($K_{D,rep}$) of the reporter ligand (2OG) is known.

Using the methods of Morton *et al.*⁵⁹ and Dalvit *et al.*,³⁹ the apparent dissociation constant of the inhibitor ($K_{D,app}$; which is defined as the free concentration of the inhibitor when 50% of the enzyme is bound with the inhibitor) can be obtained by non-linear curve fitting of the titration data (Figure 2b and 2c) according to the following equation:

$$K_{D,app} = \frac{[E_T] \times [L] - [E_T] \times [EL] + [EL]^2 - [L] \times [EL]}{[EL]} \quad (2)$$

In which $[E_T]$ is the total enzyme concentration, $[L]$ is the concentration of the inhibitor and $[EL]$ is the concentration of the enzyme-inhibitor complex.

From the experimentally determined $K_{D,app}$, it is possible to derive the K_D of an inhibitor from knowledge of the dissociation constant of the reporter ligand ($K_{D,rep}$). However, because the concentration of the reporter ligand used in this assay is comparable to the total enzyme concentration, the Cheng-Prusoff correlation⁶⁰ is not valid due to its assumption of a large excess of reporter ligand. Instead, we used the correlation described by Nikolovska-Coleska *et al.*⁶¹ and Cer *et al.*⁶² which considers all the variables including enzyme and reporter ligand concentrations. A full description of the data treatment can be found in Supplementary Information (Figure legend of Supplemental Figures S5).

In order to obtain an accurate K_D for a ligand of interest, it is crucial that the K_D value of the reporter ligand is measured accurately. The K_D values of 2OG with PHD2 (900 nM) and FIH (4 μ M) used in this study were obtained using the solvent water relaxation method,³¹ in which the native Fe^{II} was replaced by the paramagnetic Mn^{II}. The binding constants of 2OG

to PHD2-Mn^{II} (900 nM) and PHD2-Zn^{II} (<2 μ M obtained by ¹H-¹⁵N heteronuclear single quantum correlation (HSQC) titration; Supplemental Figure S2) were essentially the same.

The K_D values for the inhibitors tested are summarised in Table 1. The K_D values obtained for NOG with PHD2 and FIH are 3 μ M and 4 μ M respectively (Supplemental Figures S5 and S6), which are comparable to the reported K_D values (3 μ M for PHD2 and 2 μ M for FIH).^{31,63} In agreement with the result obtained from the single concentration screening and previous reported work,⁵² BIQ was found to be more potent against PHD2 than FIH: the K_D obtained for BIQ with PHD2 is 80 nM (Supplemental Figure S7), which is ~1800 times lower than the value obtained with FIH (140 μ M; Supplemental Figure S8). The K_D values obtained for 5C8HQ are 60 μ M and 30 μ M with PHD2 and FIH respectively (Supplemental Figures S9 and S10), supporting the result obtained from the single concentration screening (Figure 4) and are comparable to reported IC₅₀ values (15 μ M for PHD2 and 20 μ M for FIH).⁵⁴

Titration were also conducted for BNS and 2,4-PDCA with PHD2. The K_D obtained for BNS is 100 nM (Supplemental Figure S11). BNS is reported as a potent PHD2 inhibitor (IC₅₀ 20 nM),⁵⁸ and the low K_D value obtained here confirmed that it is a very strong binder to PHD2 and that it binds in the 2OG binding pocket. The K_D obtained for 2,4-PDCA is 420 nM (Supplemental Figure S12), supporting previous inhibition studies which reported 2,4-PDCA as a weaker inhibitor than BIQ, but more potent than NOG.⁵³

HIF hydroxylases and TCA cycle intermediates

After demonstrating that this method is applicable for generic 2OG oxygenase inhibitors, we conducted screening against tricarboxylic acid (TCA) cycle intermediates (except succinyl coenzyme A; Supplemental Figure S13). Information on the binding of TCA cycle intermediates to HIF hydroxylases (and other 2OG oxygenases) is of biological interest because there is evidence that TCA cycle intermediates may influence the regulation of HIF hydroxylase activities or other 2OG oxygenases.^{22,64-67}

At an inhibitor concentration of 400 μ M, the only TCA cycle intermediate that shows significant 2OG displacement is fumarate with PHD2 (~25% 2OG displacement; Supplemental Figure S14). This is in agreement with our previous inhibition data, which found that fumarate is the only compound amongst TCA cycle intermediates that inhibits PHD2 in the micromolar range.²² A titration was also conducted for fumarate with PHD2. The K_D value obtained was 200 μ M (Table 1 and Supplemental Figure S15; Reported IC₅₀ = 220 μ M²²), confirming it is a weak PHD2 binder, although its role may become more significant, for instance, in cancers that are involved in the mutations of fumarate hydratase where fumarate concentrations is reported to exceed 10 mM.⁶⁸⁻⁷⁰

Previous inhibition data also suggested succinate and isocitrate may be weak PHD2 inhibitors, with the IC₅₀ values greater than 10 mM.^{22,65} Our results suggest that, at least at the concentration that we used (40 fold molar excess), no 2OG displacement by these compounds was observed (Supplemental Figure S14). Under our screening conditions, citrate, isocitrate and malate also did not appear to displace 2OG in solution (Supplemental Figure S14), although previous non-denaturing ESI-MS data suggested they form binary

complexes with PHD2 but not ternary complexes with PHD2 and its peptidyl substrate.²² These data suggest that citrate, isocitrate and malate may not bind to the 2OG binding pocket, but could form ‘non-specific’ weak binding interactions to PHD2 (possibly to the peptidyl substrate binding site). Ascorbate, a commonly-used reducing agent in 2OG oxygenase activity assays,⁷¹ does not appear to compete with 2OG. In agreement with previous inhibition data,²² none of the TCA cycle intermediates appeared to bind substantially to FIH (Supplemental Figure S14).

Using [¹³C]-2OG as reporter ligand

One potential problem associated with CPMG-edited ¹H NMR screening is signal overlap of the reporter and ligand resonances such that it is not possible to reliably monitor reporter ligand recovery in response to its displacement. In order to address this issue, we investigated the use of commercially available [1,2,3,4-¹³C₄]-labelled 2OG as an alternative reporter ligand (Supplemental Figure S16), using the [¹³C]-label for spectral editing (Supplemental Figure S17). However, aside from cost, the penalty of using [¹³C] for editing is a loss of sensitivity, and a loss of additional *T*₂ filtering (as in the CPMG *T*₂-edited experiments) to distinguish the free and bound ligands. In order to tackle this, the 1D ¹H-¹³C HSQC was chosen in favour of direct carbon observation.⁷² A ¹³C selective version of the 1D ¹H-¹³C HSQC experiment was used to ensure clean selection of the 2OG CH₂ resonance, in which a Q3 shaped 180° pulse was applied as the selective inversion pulse in the INEPT transfer stages. In general, a slightly higher concentration of protein and 2OG was used so that a reasonable signal-to-noise ratio can be achieved whilst keeping the experiment time relatively short (<30 minutes).

By applying selective ¹³C irradiation on C-4 of 2OG (30.5 ppm), it was possible to selectively observe free 2OG (Supplemental Figure S18). Titrations were conducted for BNS and NOG with PHD2 (Supplemental Figure S19), whereby the *K*_D values obtained (~110 nM for BNS and ~2 μM for NOG; Supplemental Figures S20 and S21) were comparable to those obtained using CPMG-edited ¹H NMR (100 nM for BNS and 3 μM for NOG), thus demonstrating its potential use as an alternative approach in case of signal overlap.

Application to other 2OG oxygenases

In order to test the wider applicability of the 2OG displacement method across the 2OG oxygenase family, we investigated qualitative binding with the histone demethylase F-box and leucine-rich repeat protein 11 (FBXL11; EC 1.14.11.27) using CPMG-edited ¹H NMR.⁷³ Histone lysine methylation status is linked to cancer,⁷⁴ and therefore a reliable screening assay would be useful for future inhibitor development against FBXL11 and related 2OG dependent demethylases such as Jimonji (JmjC) domain containing enzymes.

In experiments with FBXL11, excess enzyme was found to be needed to ensure most of the 2OG signal was attenuated (Supplemental Figure S22), indicating FBXL11 is a relatively weak 2OG binder. Single point screening experiments (at 800 μM inhibitor concentration) were performed using the five inhibitors tested with PHD2 (Figure 3). The results reveal that BNS and 2,4-PDCA, two potent PHD2 inhibitors, are relatively strong binders to FBXL11

(~90% and ~70% 2OG displacement respectively; Supplemental Figure S23). NOG and 5C8HQ are also binders of FBXL11 (~30% and ~65% 2OG displacement respectively; Supplemental Figure S23). Interestingly, the PHD2 inhibitor BIQ, does not seem to displace 2OG when tested with FBXL11 (Supplemental Figure S23). These results demonstrate the wider applicability of the 2OG displacement assay across 2OG oxygenases, and will help to provide a basis for future FBXL11 inhibitor development.

Application in inhibitor discovery

Finally, we have applied the 2OG reporter method to inhibitor discovery for hit validation. Protein-directed dynamic combinatorial chemistry (DCC), which is related to the fragment based drug discovery (FBDD) method, is an emerging technique for inhibitor discovery.⁷⁵⁻⁸⁰ DCC is an approach to the generation and identification of protein ligands *via* reversible inter-conversions of simple building blocks in the presence of a target protein template (Supplemental Figure S24). Previously, we have successfully applied protein-directed DCC to identify ligands produced by reversible boronate ester formation that led to novel nanomolar inhibitors for PHD2.^{81,82} This work involved the use of a boronic acid 'scaffold', which binds in the 2OG binding pocket and is a weak inhibitor of PHD2. However, upon addition of appropriate diols, boronate esters form reversibly, which can bind tightly to PHD2 (Supplemental Figure S25).⁸²

As a proof-of-principle study, the boronic acid scaffold and the reported diol hits were subjected to the NMR reporter analyses using CPMG-edited ¹H NMR (Supplemental Figure S25).⁸² At 50 μ M concentration, the boronic acid appeared to cause ~50% 2OG displacement (Figure 5), confirming it is a weak binder to PHD2. A slightly higher concentration (150 μ M) of diols (than the boronic acid scaffold) was used to ensure the generation of the boronate ester species. In the absence of the boronic acid, none of the diols appeared to displace 2OG from PHD2 (Figure 5). However, in the presence of the boronic acid and the diols, a significant improvement in 2OG displacement was observed (~75% to ~85% 2OG displacement; Figure 5), in agreement with the results obtained from non-denaturing electrospray ionisation mass spectrometry (ESI-MS).⁸² As negative controls, in the presence of the boronic acid scaffold and butane-2,3-diol, no improvement in 2OG displacement was observed (Figure 5), and in the presence of the boronic acid scaffold and catechol, only a moderate improvement in 2OG displacement was observed (~60%; Figure 5), again in good agreement with the ESI-MS result.⁸²

These results validate previous screening results obtained by non-denaturing ESI-MS⁸² and support the proposal that it is the boronate ester species that are responsible for the improvement in binding and inhibition, and demonstrate the potential of the reporter method for hit identification.

Conclusions

In this study we have demonstrated the development and application of reporter ligand ¹H NMR analyses to the study of ligand binding to 2OG oxygenases, using the natural cosubstrate 2OG as a reporter ligand. This method relies on the displacement of 2OG on binding of competitive ligand and direct ¹H NMR observation of unbound 2OG. It can

provide site-specific binding information, and may also provide an accurate determination of dissociation constants for both strong and weak binders, which is otherwise difficult to estimate using other ligand-based NMR techniques. We have further shown that signal overlap, a problem associated with many ^1H NMR based screening methods, can be overcome by the use of commercially available $[1,2,3,4\text{-}^{13}\text{C}_4]$ -labelled 2OG combined with a 1D selective HSQC experiment for editing.

The reporter method has been applied to different members of the 2OG oxygenase superfamily, including HIF hydroxylases (PHD2 and FIH) and histone demethylases (FBXL11) and is proposed as a generic NMR based screening method for this superfamily. A caveat of the method is that it does not employ the native metal, Fe^{II} , and it should be noted that use of an alternative metal could affect binding modes. Nevertheless, our results demonstrated a good correlation between binding data from the NMR reporter method employing Zn^{II} and that from catalytic turnover assays using native Fe^{II} . To date, most 2OG oxygenase inhibitors are designed as 2OG competitors,¹⁷ and this method can also be applied to validate the binding mode of these compounds. We have also screened and quantified the binding affinities of TCA cycle intermediates that may be involved in HIF regulation in certain cancers, and have shown that this method can be applied as a screening or validation tool in hit discovery, as exemplified by protein-directed dynamic combinatorial chemistry. Finally, we also propose that this NMR-based approach of using cofactors/cosubstrates as reporters could be more widely applied in general NMR screening assays for other superfamilies of enzymes including kinases and ATPases.

Experimental Section

Materials

Chemicals were from Acros, Alfa Aesar, Apollo Scientific, Cambridge Isotope Laboratories or Sigma-Aldrich. BNS, BIQ, 5C8HQ, NOG (Figure 3) and the 'scaffold' boronic acid (Figure 5) were synthesised by the reported methods.^{48,50,54,58,82} The purity of all compounds synthesised were 95% as determined by analytical reverse-phase HPLC (UltiMate 3000; Dionex).

Expression and Purification of PHD2, FIH and FBXL11

The catalytic domain of PHD2 (residues 181-426) and full length FIH and FBXL11 were expressed in *E. coli* and purified by metal affinity, cation exchange and size exclusion chromatography, essentially as described.^{18,63,83}

NMR experiments

NMR experiments were conducted at a ^1H frequency of 700 MHz using a Bruker Avance III spectrometer equipped with a TCI inverse cryoprobe. All experiments were conducted at 298 K. 3 mm diameter MATCH NMR tubes (Bruker) using a sample volume of 160 μL were used in all experiments. Solutions were buffered using 50 mM Tris-D11 (pH 7.5) dissolved in 90% H_2O and 10% D_2O . The pulse tip-angle calibration using the single-pulse nutation method⁸⁴ (Bruker *pulseval* routine) was undertaken for each sample.

CMPG-edited screening experiments

Samples contained 10 μM *apo*-PHD2/*apo*-FIH, 80 μM Zn^{II} , 10 μM 2OG and 400 μM potential inhibitor. In case of FBXL11, 35 μM *apo*-protein was used and inhibitors were screened at 800 μM concentration. The PROJECT-CPMG sequence ($90^\circ_x - [\tau - 180^\circ_y - \tau - 90^\circ_y - \tau - 180^\circ_y - \tau]_n - \text{acq}$) was applied as described by Augilar *et al.*⁴⁷ Typical experimental parameters were as follows: total echo time 48 ms ($\tau = 2$ ms, $n = 6$), acquisition time 2.94 seconds, relaxation delay 2 seconds, number of transients 128. Water suppression was achieved by presaturation. All experiments were conducted at 298 K.

1D selective HSQC

Samples contained 150 μM *apo*-PHD2, 200 μM Zn^{II} and 150 μM 2OG. The CLIP-HSQC sequence was used for 1D HSQC experiments (without ^{13}C decoupling).⁸⁵ Typical experimental parameters were as follows: acquisition time 0.58 seconds, relaxation delay 2 seconds, number of transients 256–1600. The $^1J_{\text{CH}}$ was set to 145 or 160 Hz. A 6.8 ms Q3.1000 180 degree pulse was used and ^{13}C selective irradiation was applied at 30.5 ppm (2OG C-4). All experiments were conducted at 298 K.

Dissociation constant determination

Experiments were conducted under the same conditions as described above, except for varying inhibitor concentrations. Inhibitor stock solution of 0.5–1.5 μL was titrated into NMR tubes. $K_{\text{D,app}}$ curves were fitted using OriginPro 8.0 (OriginLab, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

2,4-PDCA	2,4-pyridine dicarboxylic acid
2OG	2-Oxoglutarate
5C8HQ	5-carboxy-8-hydroxyquinoline
BIQ	bicyclic isoquinolinyl inhibitor
BNS	bicyclic naphthalenylsulfonyl inhibitor
CD	circular dichroism
CPMG	Carr-Purcell-Meiboom-Gill
DCC	dynamic combinatorial chemistry
ESI-MS	electrospray ionisation mass spectrometry

EPR	electron paramagnetic resonance
FBDD	fragment based drug discovery
FIH	factor inhibiting hypoxia inducible factor
FBXL11	F-box and leucine-rich repeat protein 11
NOG	<i>N</i> -oxalylglycine
HSQC	heteronuclear single quantum correlation
HIF	hypoxia inducible factor
ITC	isothermal titration calorimetry
NMR	nuclear magnetic resonance
PHD2	prolyl hydroxylase domain containing enzyme isoform 2
PROJECT	periodic refocusing of J evolution by coherence transfer
SPR	surface plasmon resonance
STD	saturation transfer difference
TCA	tricarboxylic acid
waterLOGSY	water ligand observed gradient spectroscopy

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Figure 1.

Schematic representation for the NMR reporter screening method. When the reporter ligand is bound to the protein, the corresponding ^1H NMR resonance is broadened and its intensity lowered. In the presence of a competitive inhibitor, the reporter ligand is displaced from the protein binding site and its signal intensity is recovered.

Figure 2.

2OG displacement from human PHD2 as monitored by CPMG-edited ^1H analyses. (a) Single concentration screening at 400 μM inhibitor concentration. The non-binder is *cis*-aconitate; the strong binder is the bicyclic naphthalenylsulfonyl inhibitor (BNS) and the weaker binder is fumarate; (b) Titration experiment monitoring 2OG signal recovery at different inhibitor (*N*-oxalylglycine; NOG) concentrations; (c) Corresponding plot of the NOG titration data. The percentage 2OG displacement is defined in equation (1). Samples contained 10 μM apo-PHD2, 80 μM Zn^{II} , 10 μM 2OG and 50 mM Tris-D11 pH 7.5 in 90%

H₂O + 10 D₂O. The asterisk indicates the 2OG CH₂ resonance at 2.35 ppm that is being monitored.

Reporter ligand **2OG****2OG oxygenase inhibitors** **BIQ** **5C8HQ** **NOG** **2,4-PDCA** **BNS**

Figure 3.
Structures of compounds used in this study.

Figure 4.

Single concentration 2OG displacement experiments using CPMG-edited ^1H analyses. The assay mixture contained 10 μM *apo*-PHD2 or *apo*-FIH, 80 μM Zn^{II} , 10 μM (non-labelled) 2OG, 400 μM inhibitor and 50 mM Tris-D11 pH 7.5 in 10% D_2O and 90% H_2O . The errors shown are the standard derivation from three separate measurements. The percentage 2OG displacement is defined in equation (1). n.d. = not determined.

Figure 5.

The addition of certain diols improves the binding of the boronic acid scaffold to PHD2- Zn^{II} , as reflected by the improvement in 2OG displacement as monitored by the reporter ligand screening method using CPMG-edited ^1H analyses. The final sample mixtures contained 10 μM *apo*-PHD2, 80 μM Zn^{II} , 10 μM (non-labelled) 2OG, 50 μM boronic acid and 150 μM diol where applicable. Solutions were buffered using 50 mM Tris-D11 (pH 7.5) in 90% H_2O and 10% D_2O . The percentage 2OG displacement is defined in equation (1).

Table 1

Binding constants (K_D) for the catalytic domain of human prolyl hydroxylase isoform 2 (PHD2) and factor inhibiting hypoxia inducible factor (FIH) measured by NMR in this study. The errors shown are the standard derivation from three separate measurements. The obtained K_D values are based on the K_D values for 2OG binding to PHD2-Mn^{II} and FIH-Mn^{II}, which are 900 nM and 4 μ M respectively. Reported IC₅₀ and K_D values were obtained from references 22, 31, 53, 54 and 58.

Compound	K_D	Reported IC ₅₀ (or K_D)
<i>Binding to PHD2</i>		
NOG	$3 \pm 1 \mu\text{M}$	20 μM (3 μM)
BIQ	$80 \pm 5 \text{ nM}$	70 nM (< 1 μM)
5C8HQ	$60 \pm 5 \mu\text{M}$	15 μM
BNS	$100 \pm 10 \text{ nM}$	20 nM (< 1 μM)
2,4-PDCA	$420 \pm 130 \text{ nM}$	2 μM
fumarate	$200 \pm 70 \mu\text{M}$	220 μM
<i>Binding to FIH</i>		
NOG	$4 \pm 1 \mu\text{M}$	50 μM (2 μM)
BIQ	$140 \pm 10 \mu\text{M}$	> 25 μM
5C8HQ	$30 \pm 10 \mu\text{M}$	20 μM