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Reporter Ligand NMR Screening Method for 2-Oxoglutarate Oxygenase Inhibitors

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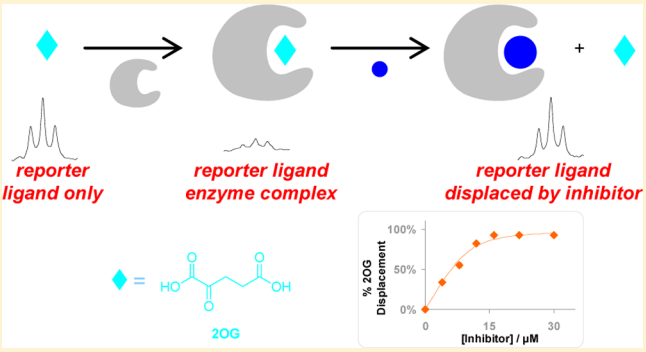
Supporting Information

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 hydroxylases and histone demethylases, and K_D values were determined for inhibitors that compete with 2OG at the metal center. This technique is also useful as a screening or validation tool for inhibitor discovery, as exemplified by work with protein-directed dynamic combinatorial chemistry.



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 anemia.[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 utilize 2OG and oxygen as cosubstrates to catalyze two-electron oxidation reactions (mostly hydroxylations), with the release of succinate and carbon dioxide as coproducts (Figure S1, Supporting Information). This property has led to the development of several generic activity-based assays (for reviews see refs 17 and 18). However, activity-based assays are not always well-suited to the initial stages of medicinal chemistry, for example, 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 nondenaturing electrospray ionization mass spectrometry (ESI-MS; for examples see refs 19−23), protein X-ray crystallography (for reviews see refs 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, nondenaturing ESI-MS has been used by our group as a simple and rapid primary screening method for 2OG oxygenase inhibitors. However, because noncovalent 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 labeled 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 nonspecific binding,[35] 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] fluorine,[42] and carbon,[43] have been proposed for reporter screening by NMR.

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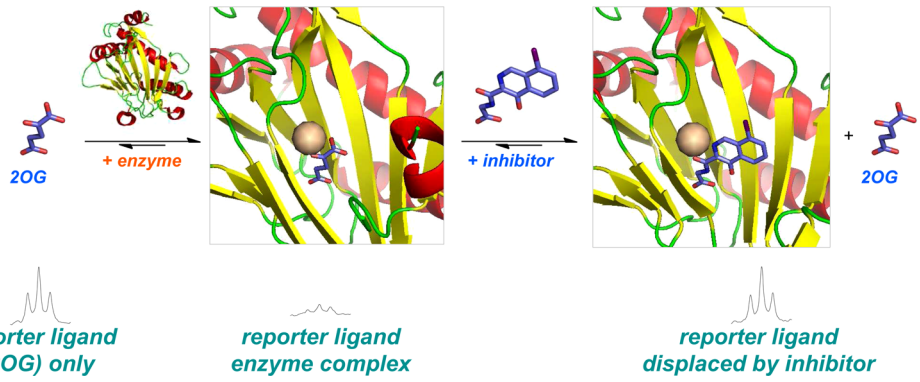

Figure 1. Schematic representation for the NMR reporter screening method. When the reporter ligand is bound to the protein, the corresponding ^{1}H NMR resonance is broadened, and its intensity is lowered. In the presence of a competitive inhibitor, the reporter ligand is displaced from the protein binding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

We reasoned that 2OG is a potentially good reporter ligand candidate for generic NMR binding assays for 2OG oxygenases because they all utilize it as a cosubstrate. Using unlabeled and commercially available [1,2,3,4-$^{13}C_4$]-labeled 2OG ([^{13}C]-2OG), we have optimized 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 HIF hydroxylases, in particular, the prolyl hydroxylase domain containing enzyme isoform 2 (PHD2; EC 1.14.11.29; for reviews see refs 6−8) and factor inhibiting HIF (FIH; EC 1.14.11.30; for reviews see refs 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 catalyze 2OG turnover in the absence of substrates, native Fe^{II} was substituted with the diamagnetic Zn^{II} in order to block enzyme-catalyzed 2OG turnover and to avoid the oxidation of Fe^{II} to paramagnetic Fe^{III}. In the assays, an 8-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 substantially affected by the use of Zn^{II} as the active site metal (see below; Figure S2, Supporting Information).

Both PHD2 and FIH form stable complexes with 2OG that saturate at a ∼1:1 ratio (Figure S3, Supporting Information).[23,31] Under these conditions, the ^{1}H NMR spectra were dominated by the protein resonances (Figure S4, Supporting Information). 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 (Figure S4, Supporting Information). 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 ^{1}H resonances was observed due to a high ^{1}H transverse relaxation rate in the bound state, so the resultant CPMG-edited ^{1}H 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 the bulk solution, leading to sharpening of the signals and recovery of the 2OG signal intensity (Figure 2a). The simplicity and sensitivity of the experiment make 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

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

where I_{2OG} is the integral of the 2OG ^{1}H signal in the presence of both the inhibitor and the protein, $I_{2OG(0)}$ is the integral of 2OG in the presence of the protein but in the absence of the inhibitor, and $I_{2OG(blank)}$ 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), $I_{2OG(0)}$ 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 nonreactive 2OG analogue, with a NH group replacing the CH_2 at the C-3 position of 2OG. It is a relatively nonselective 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

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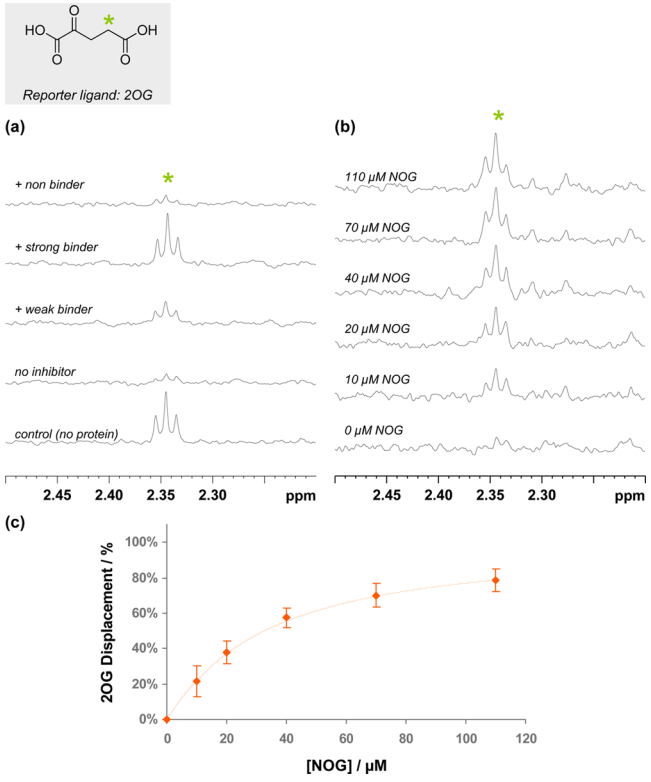

Figure 2. 2OG displacement from human PHD2 as monitored by CPMG-edited ^{1}H analyses. (a) Single concentration screening at 400 μM inhibitor concentration. The nonbinder is *cis*-aconitate; the strong binder is the bicyclic naphthalenylsulfonyl inhibitor (BNS), and the weaker binder is fumarate; (b) titration experiment monitoring 2OG signal recovery at different inhibitor (*N*-oxalyglycine; NOG) concentrations; (c) corresponding plot of the NOG titration data. The percentage 2OG displacement is defined in eq 1. Samples contained 10 μM apo-PHD2, 80 μM Zn^{II}, 10 μM 2OG, and 50 mM Tris-D11 pH 7.5 in 90% H_2O and 10% D_2O. The asterisk indicates the 2OG CH_2 resonance at 2.35 ppm that is being monitored.

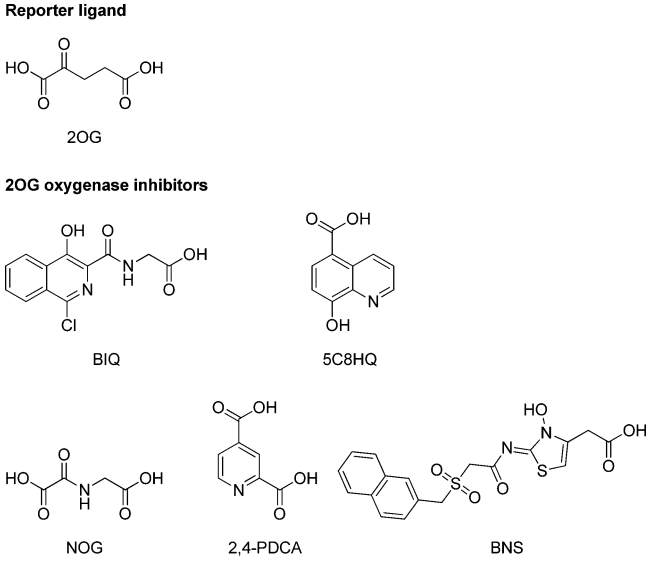

Figure 3. Structures of compounds used in this study.

with 5-carboxy-8-hydroxyquinoline (5C8HQ; Figure 3),[54] which is 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: 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).

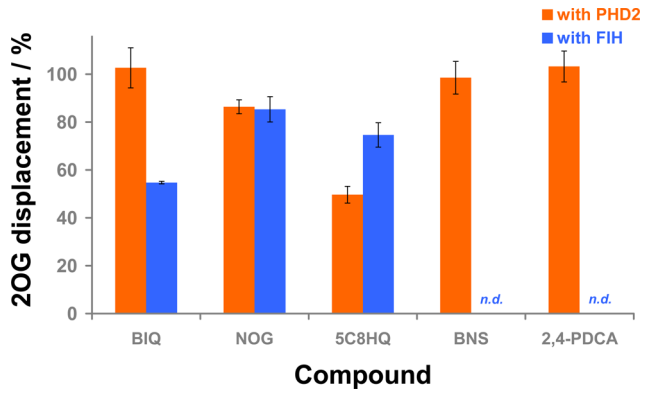

Figure 4. Single concentration 2OG displacement experiments using CPMG-edited ^{1}H analyses. The assay mixture contained 10 μM apo-PHD2 or apo-FIH, 80 μM Zn^{II}, 10 μM (nonlabeled) 2OG, 400 μM inhibitor, and 50 mM Tris-D11 pH 7.5 in 10% D_2O and 90% H_2O. The errors shown are the standard deviation from three separate measurements. The percentage 2OG displacement is defined in eq 1. n.d. = not determined.

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.

By 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 nonlinear curve fitting of the titration data (Figure 2b,c) 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 correlation[60] 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 the Supporting Information (Figure legend of Figure S5).

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In order to obtain an accurate K_D value 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 μM) used in this study were obtained using the solvent water relaxation method,[31] in which native Fe^{II} was replaced by paramagnetic Mn^{II}. The binding constants of 2OG to PHD2-Mn^{II} (900 nM) and PHD2-Zn^{II} (<2 μM obtained by $^1H-^{15}N$ heteronuclear single quantum correlation (HSQC) titration; Figure S2, Supporting Information) were essentially the same.

The K_D values for the inhibitors tested are summarized in Table 1. The K_D values obtained for NOG with PHD2 and FIH

Table 1. Binding Constants (K_D) for the Catalytic Domain of Human PHD2 and FIH Measured by NMR in This Study[a]

compound	K_D	reported IC_{50} (or K_D)
	Binding to PHD2	
NOG	3 ± 1 μM	20 μM (3 μM)
BIQ	80 ± 5 nM	70 nM (<1 μM)
5C8HQ	60 ± 5 μM	15 μM
BNS	100 ± 10 nM	20 nM (<1 μM)
2,4-PDCA	420 ± 130 nM	2 μM
fumarate	200 ± 70 μM	220 μM
	Binding to FIH	
NOG	4 ± 1 μM	50 μM (2 μM)
BIQ	140 ± 10 μM	>25 μM
5C8HQ	30 ± 10 μM	20 μM

[a]The errors shown are the standard deviation 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 μM, respectively. Reported IC_{50} and K_D values were obtained from refs 22, 31, 53, 54, and 58.

are 3 and 4 μM, respectively (Figures S5 and S6, Supporting Information), which are comparable to the reported K_D values (3 μM for PHD2 and 2 μM 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 (Figure S7, Supporting Information), which is ~1800 times lower than the value obtained with FIH (140 μM; Figure S8, Supporting Information). The K_D values obtained for 5C8HQ are 60 and 30 μM with PHD2 and FIH, respectively (Figures S9 and S10, Supporting Information), supporting the result obtained from the single concentration screening (Figure 4) and are comparable to reported IC_{50} values (15 μM for PHD2 and 20 μM for FIH).[54]

Titrations were also conducted for BNS and 2,4-PDCA with PHD2. The K_D value obtained for BNS is 100 nM (Figure S11, Supporting Information). BNS is reported as a potent PHD2 inhibitor (IC_{50} 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 value obtained for 2,4-PDCA is 420 nM (Figure S12, Supporting Information), supporting previous inhibition studies that reported 2,4-PDCA as a weaker inhibitor than BIQ but more potent than NOG.[53]

HIF Hydroxylases and Tricarboxylic Acid Cycle Intermediates. After demonstrating that the method is applicable for generic 2OG oxygenase inhibitors, we conducted screening against tricarboxylic acid (TCA) cycle intermediates (except succinyl coenzyme A; Figure S13, Supporting Information). 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 μM, the only TCA cycle intermediate that shows significant 2OG displacement is fumarate with PHD2 (~25% 2OG displacement; Figure S14, Supporting Information). This is in agreement with our previous inhibition data, which found that fumarate is the only compound among 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 μM (Table 1 and Figure S15, Supporting Information; reported IC_{50} = 220 μM[22]), confirming it is a weak PHD2 binder, although the role of fumarate-mediated PHD inhibition may become significant, for instance, in cancers that are involved in the mutations of fumarate hydratase where fumarate concentrations are reported to exceed 10 mM.[68−70]

Previous inhibition data also suggested succinate and isocitrate may be weak PHD2 inhibitors, with IC_{50} values greater than 10 mM.[22,65] Our results suggest that, at least at the concentration used (40-fold molar excess), no 2OG displacement by these compounds was observed (Figure S14, Supporting Information). Under our screening conditions, citrate, isocitrate, and malate also did not appear to displace 2OG in solution (Figure S14, Supporting Information), although previous nondenaturing 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 "nonspecific" 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 (Figure S14, Supporting Information).

Using [^{13}C]-2OG as a 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-$^{13}C_4$]-labeled 2OG as an alternative reporter ligand (Figure S16, Supporting Information), using the [^{13}C]-label for spectral editing (Figure S17, Supporting Information). However, aside from cost, the penalty of using [^{13}C] 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 issue, one-dimensional (1D) $^1H-^{13}C$ HSQC was chosen in favor of direct carbon observation.[72] A ^{13}C selective version of the 1D $^1H-^{13}C$ HSQC experiment was used to ensure clean selection of the 2OG CH_2 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 while keeping the experiment time relatively short (<30 min).

By applying selective ^{13}C irradiation on C-4 of 2OG (30.5 ppm), it was possible to selectively observe free 2OG (Figure S18, Supporting Information). Titrations were conducted for BNS and NOG with PHD2 (Figure S19, Supporting

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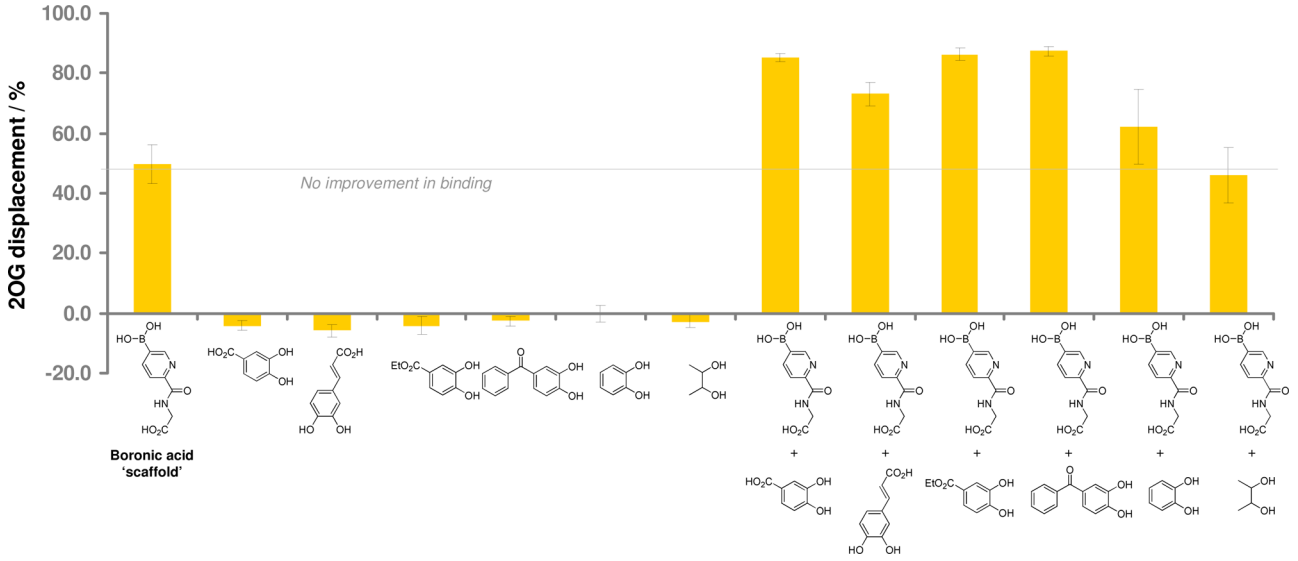

Figure 5. Addition of certain diols improves the binding of the boronic acid scaffold to PHD2-Zn^{II}, as reflected by the improvement in 2OG displacement as monitored by the reporter ligand screening method using CPMG-edited ^{1}H analyses. The final sample mixtures contained 10 μM apo-PHD2, 80 μM Zn^{II}, 10 μM (nonlabeled) 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 eq 1.

Information), whereby the K_D values obtained (~110 nM for BNS and ~2 μM for NOG; Figures S20 and S21, Supporting Information) were comparable to those obtained using CPMG-edited ^{1}H 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 ^{1}H 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 (Figure S22, Supporting Information), indicating FBXL11 is a relatively weak 2OG binder. Single-point screening experiments (at 800 μM inhibitor concentration) were performed using five of the 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; Figure S23, Supporting Information). NOG and 5C8HQ are also binders of FBXL11 (~30% and ~65% 2OG displacement, respectively; Figure S23, Supporting Information). Interestingly, the PHD2 inhibitor BIQ does not seem to displace 2OG when tested with FBXL11 (Figure S23, Supporting Information). 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 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 interconversions of simple building blocks in the presence of a target protein template (Figure S24, Supporting Information). 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 (Figure S25, Supporting Information).[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 ^{1}H NMR (Figure S25, Supporting Information).[82] At 50 μM 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 μM) 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 nondenaturing 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 nondenaturing ESI-MS[82] and support the proposal that it is the boronate ester species that are responsible for the improvement in binding and inhibition and validate the potential of the reporter method for hit identification.

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CONCLUSIONS

In this study, we have demonstrated the development and application of reporter ligand ^{1}H 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 ^{1}H 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 ^{1}H NMR based screening methods, can be overcome by the use of commercially available [1,2,3,4-$^{13}C_4$]-labeled 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 DCC. 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 synthesized by the reported methods.[48,50,54,58,82] The purity of all compounds synthesized were ≥95% as determined by analytical reverse-phase high-performance liquid chromatography (UltiMate 3000; Dionex).

Expression and Purification of PHD2, FIH, and FBXL11. The catalytic domains of PHD2 (residues 181–426) and FBXL11 (residues 1–517) and full length FIH were expressed in *Escherichia coli* and purified by metal-affinity, cation-exchange, and size-exclusion chromatographies, essentially as described.[18,63,83]

NMR Experiments. NMR experiments were conducted at a ^{1}H frequency of 700 MHz using a Bruker Avance III spectrometer equipped with a TCI inverse cryoprobe. All experiments were conducted at 298 K. MATCH NMR tubes (3 mm diameter, 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 method[84] (Bruker *pulsecal* 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 the case of FBXL11, 35 μM apo-protein was used, and inhibitors were screened at 800 μM concentration. The PROJECT-CPMG sequence ($90°_x$–[τ–$180°_y$–τ–$90°_y$–τ–$180°_y$–τ]$_n$–acq) was applied as described by Augilar et al.[47] Typical experimental parameters were as follows: total echo time, 48 ms (τ = 2 ms; n = 6); acquisition time, 2.94 s; relaxation delay, 2 s; 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 s; relaxation delay, 2 s; number of transients, 256–1600. The $^1J_{CH}$ was set to 145 or 160 Hz. A 6.8 ms Q3 180° 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. An inhibitor stock solution of 0.5–1.5 μL was titrated into NMR tubes. $K_{D,app}$ curves were fitted using OriginPro 8.0 (OriginLab, USA).

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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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 ionization 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, *N*-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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