Supporting Information

for

Affinity-Based Fluorescence Polarization Assay for High-Throughput Screening of Prolyl Hydroxylase 2 Inhibitors

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I. Supplementary Figures

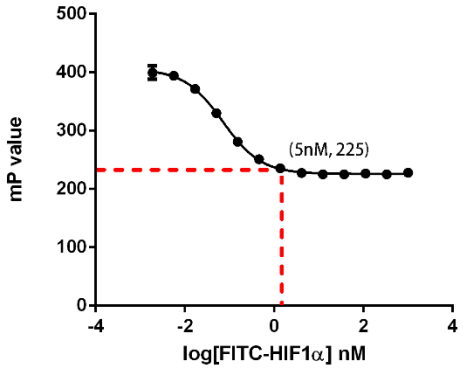

Figure S1. Determine of the optimal concentration of the free probe for fluorescence polarization assay by varying the concentration of the free probe. The experiment was using 12 concentrations of FITC-HIF1α in 3-fold serial dilutions from 1 μM. To avoid the use of high amounts of tracer but also to eliminate possible inconsistencies in concentration measurements, a FITC-HIF1α concentration of 5 nM appeared optimal for use in competitive assays.

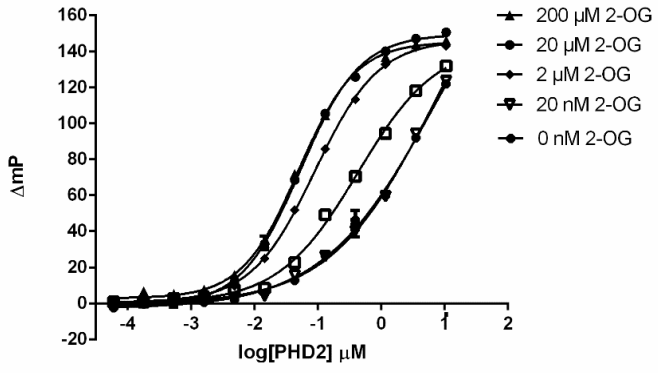

Figure S2. Optimization of the binding conditions for PHD2 and FITC-HIF1α with different concentration of 2-OG and 100 μM Mn^{II}. At the concentration of 20 μM, the binding of PHD2 and Fitc-HIF-α was saturated.

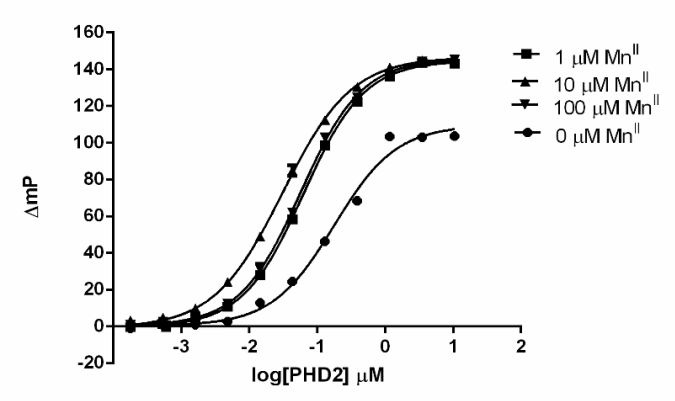

Figure S3. Optimization of the binding conditions for PHD2 and FITC-HIF1α with different concentration of Mn^{II} and 20 μM 2-OG. The binding affinity was increased when the concentration reduced to 10 μM from 100 μM. Further reducing the use of Mn^{II} to 1 μM led to an obvious loss in binding affinity. So we chose a suitable concentration at 10 μM in the following test.

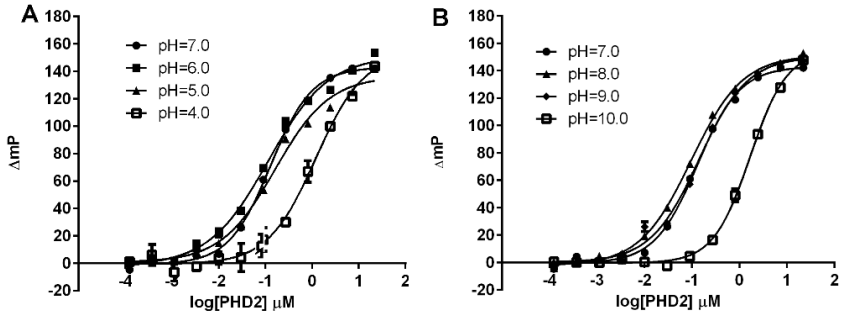

Figure S4. Evaluation of the influence of pH on the assay performance. Fluorescence polarization signals were measured from pH 4 to pH 10, as shown in the figure there is virtually no influence of pH on binding properties of PHD2 and fluorescent HIF1α peptide at pH 6-9; at the pH<6 or pH>9, the binding activity of the enzyme was decreased.

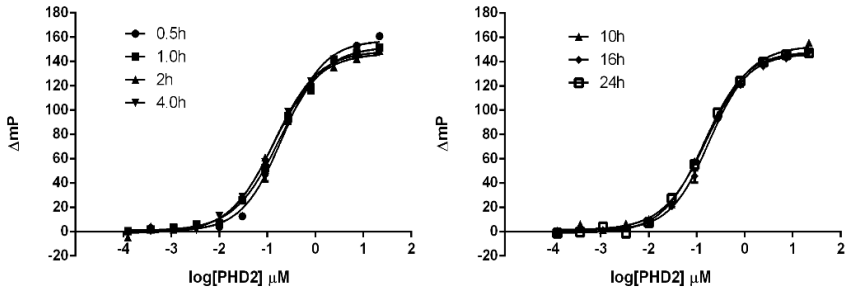

Figure S5. Binding of probe to PHD2 in the presence of Mn^{II} is highly stable over time. Fluorescence polarization signals were measured at various time points over 24 hours. Once binding reached equilibrium (approximately 1 hours), signals stayed stable over the course of at least 22 hours.

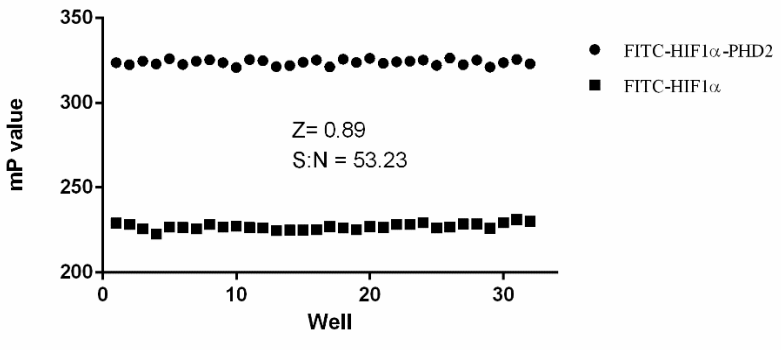

Figure S6. Evaluation of PHD2 fluorescence polarization assay performance

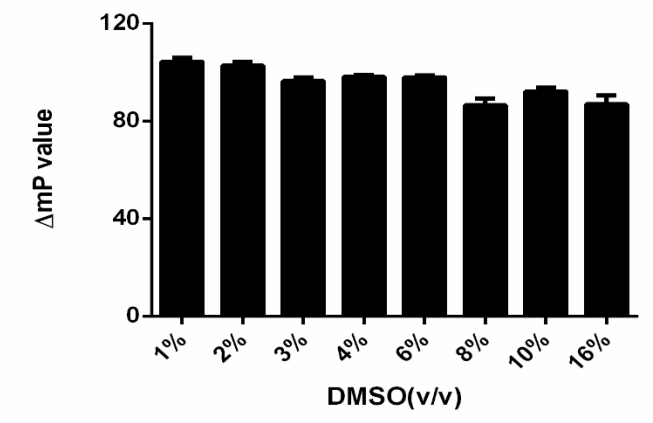

Figure S7. Effect of DMSO on binding experiments

Table S1. The assay methods and IC_{50} of the reported PHD2 inhibitors

Compounds	Reported assay, IC_{50}	Our assay, IC_{50}
FG-2216	MOL-TOF MS, 300 nM [S2]	424.2 ±1.5 nM
FG-4592	—	591.4 ±13.0 nM
1	AlphaScreen, 650 nM [S3]	890.4 ±5.6 nM
BAY-85-3934	Homogeneous time-resolved fluorescence assay, 280 nM [S4]	876.3 ±11.2 nM

II. Supplementary Methods

1. General methods.

PHD2 (181-426) ,which is reported to be similarly active to the full-length PHD2 (Choi et al., 2005),[S1] was from Nanjing zoombio biotechmology, and FITC-HIF1α (556-574) was from Shanghai Apeptide Co., Ltd. Reagents were obtained from Aldrich or Aladdin in the highest purity available and used as supplied. Fluorescence assay was recorded by a SpectraMax GeminiXS (Molecular Devices, Sunnyvale, CA. Excitation at the wavelength of 485 ± 25 nM and emission at the wavelength of 535 ± 25 nM).

2. General fluorescence polarization methods.

Fluorescence polarization experiments were performed in 384-well, flat bottom, black assay plates (#3575, Corning) at a final volume of 60 μL. The final assay buffer contained 5 nM Fitc-HIF1α 556-574, 0.1 μM PHD2 (181-426), 10 mM Hepes, pH 7.4, 150 mM NaCl, 10 μM MnCl2, 20μM of 2-OG, 0.05% Tween-20, and less than 1% DMSO (except for DMSO tolerance experiments, in which different DMSO concentrations were used as indicated). All plates measured in fluorescence polarization assays were incubated for a minimum of 30 min at RT, and then polarization was measured from the top of the well with a SpectraMax GeminiXS plate reader with polarized filters and optical modules for fluorescein (λex = 485 nM ± 25 nM, λem = 535 nM ± 25 nM). All measurements were performed in duplicate except

for Z´factor determination assays in which 30 replicates were used in each group. Standard error of the mean (SEM) values, were calculated by dividing the sample standard deviation by the square root of the sample size and are recorded as ± values for IC_{50}.

3. Dimethyl sulfoxide tolerance.

Increasing concentrations of DMSO (1%–16% of assay volume) were added to the reaction mixture containing 5 nM Fitc-HIF1α 556-574 and 200 nM PHD2 (181-426) protein. The reaction mixture was incubated at RT for 60 min and then total fluorescence and fluorescence polarization measurements were taken.

4. Assay accuracy and precision.

Z' factor statistical experiments were done to determine assay robustness in a high-signal group (Fitc-HIF1α 556-574 bound to PHD2 (181-426) and a low-signal group (Fitc-HIF1α 556-574 only) in 30 replicates and experiments were repeated 2 more times on different days. The reported Z´factor for this assay is an average of these 3 experiments. The Z´ factor was calculated using Equation 1, where σ_b and σ_f are the standard deviations of the high- and low-signal groups, respectively, and μ_b and μ_f are the means of the high and low signal groups, respectively.

$$Z' = 1 - 3(\sigma_b - \sigma_f) / |\mu_b - \mu_f| \quad (1)$$

5. Competitive binding fluorescence polarization assays.

Competitive binding experiments were conducted using assay buffer and 0.6 μL competitive binding compound (in DMSO) to give a final concentration of 1% DMSO in a total volume of 60 μL. Dose-dependent experiments were performed in the same manner using at least 10 concentrations of compound in 3-fold serial dilutions from 100 μM. For each assay, negative controls (equivalent to 0% displacement) contained the fluorescent ligand, PHD2, and 0.6 μL DMSO, and blank controls contained only the fluorescent ligand and DMSO. The reaction mixture was incubated at RT for 60 min and then total fluorescence and fluorescence polarization measurements were taken. Percent inhibition was calculated using Equation 2 where mP_{free} is the signal for the free probe (blank control) and mP_{bound} is the signal for the bound probe (negative control).

$$\%\ inhibition = 100 * (1 - (mP_{bound} - mP) / (mP_{bound} - mP_{free})) \quad (2)$$

The IC_{50} was determined for duplicate measurements by nonlinear least-squares analysis using GraphPad Prism 5.0.

III. References

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(S2) Chowdhury R.; Candela-Lena J. I.; Chan M. C.; Greenald D. J.; Yeoh K. K.; Tian Y. M.; McDonough M. A.; Tumber A.; Rose N. R.; Conejo-Garcia A.; Demetriades M.; Mathavan S.; Kawamura A.; Lee M. K.; van Eeden F.; Pugh C. W.; Ratcliffe P. J.; Schofield C. J. Selective small molecule probes for the hypoxia inducible factor (HIF) prolyl hydroxylases. *Chem. Biol*. **2013**, *8*, 1488.

(S3) Kawamoto, R. M. Prolyl hydroxylase inhibitors and methods of use. US patent 20070299086A1, 2007.

(S4) Flamme, I.; Oehme, F.; Ellinghaus, P.; Jeske, M.; Keldenich, J.; Thuss, U. Mimicking Hypoxia to Treat Anemia: HIF-Stabilizer BAY85-3934 (Molidustat) Stimulates Erythropoietin Production without Hypertensive Effects. *PLoS One*. **2014**, *11*, e111838.