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

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S Supporting Information

ABSTRACT: Prolyl hydroxylase domain 2 (PHD2) enzyme, a Fe^{II} and 2-oxoglutarate (2-OG) dependent oxygenase, mediates key physiological responses to hypoxia by modulating the levels of hypoxia inducible factor 1- α (HIF1 α). PHD2 has been shown to have the therapeutic potentials for conditions including anemia and ischemic disease. Currently, many activity-based assays have been developed for identifying PHD2 inhibitors. Here we report an affinity-based fluorescence polarization method using FITC-labeled HIF1 α (556–574) peptide as a probe for quantitative and site-specific screening of small molecule PHD2 inhibitors.

KEYWORDS: PHD2, FITC-labeled probe, affinity-based, fluorescence polarization assay

Hypoxia is linked to human diseases such as anemia and ischemia.^{1,2} In humans, the response to hypoxia is mediated by up-regulation of the hypoxia inducible transcription factor (HIF).^{3–5} HIF is a heterodimeric transcription factor composed of an oxygen-dependent α -subunit and constitutively expressed β -subunit. Under normoxic conditions, HIF α , the regulatory subunit of HIF dimer, is constitutively produced with a half-life of approximately 5 min. It is hydroxylated by prolyl hydroxylases 1–3 (PHD1–3), recognized by von Hippel Lindau protein (pVHL), and then rapidly ubiquitinated and subsequently degraded by the 26S proteasome.⁶ PHDs are members of the dioxygenase family that require O₂, Fe^{II}, and 2-oxoglutarate (2-OG) for their catalytic activity, which are responsible for the C4 trans hydroxylation of HIF α at Pro402 and Pro564 that initiates the path to protein degradation.⁷ It is currently believed that PHD2 plays a dominant role in controlling the cellular HIF α levels.⁸ Inhibitor of PHD2 has been pursued as a promising therapy for conditions including anemia and ischemic disease.

To discover small molecules that can regulate PHD2 activity, many activity-based assays have been developed. The development of activity-based assay was based on the catalytic activity of PHD2, which utilizes 2-OG and oxygen as cosubstrates to catalyze the prolyl hydroxylations.^{9–11} This property has led to the development of several generic activity-based assays, which detected the activity by measuring the ratio of HIF α peptide and its hydroxylated product, such as fluorescence-based assay using o-phenylenediamine,¹² MALDI-TOF MS,¹³ AlphaScreen

assay,¹⁴ homogeneous time-resolved fluorescence assay,¹⁵ and fluorescence polarization assay based on HIF-von Hippel-Lindau protein-Elongin B–Elongin C (VBC) interaction.¹⁶ 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. Recently, affinity-based assays that utilize non-denaturing electrospray ionization mass spectrometry (ESI-MS),¹⁷ affinity selection mass spectroscopy assay (AS-MS),¹⁵ or nuclear magnetic resonance (NMR)¹⁸ technology have been developed for studying the binding of metal ions and small molecules with PHD2 protein. Among them, AS-MS assay and NMR assay can be used for quantitative and site-specific screening of ligand binding to PHD2, which are suitable for the early work. However, the use of high concentration of protein and compounds makes them costly and thus limits their application to high-throughput screening of PHD2 inhibitors.

Here we would like to report a validation simple method, which is called a fluorescence polarization based assay using fluorescein isothiocyanate (FITC) labeled HIF1 α (556–574) peptide as a probe. The method relies on the displacement of 2-OG and FITC-HIF1 α (556–574) on binding of competitive ligand. We have optimized the experimental conditions and demonstrated the feasibility of applying this method for high-

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Figure 1. Schematic representation of fluorescence polarization assays used to monitor the interactions between FITC-labeled HIF1 α peptide (DLDLEMLAPYIPMDDDFQL) and PHD2 protein and displacement of the peptide by small molecule.

throughput screening for small molecule PHD2 inhibitors. It has been evident that HIF1 α (556–574) peptide can bind to the catalytic domain of PHD2 in the presence of 2-OG and metal cofactors in X-ray.¹⁹ HIF1 α (556–574) peptide has also been used as substrate of PHD2 in the activity-based assays.^{14,15} In the light of these, we designed a fluorescence probe FITC-labeled HIF1 α (556–574) peptide, which can be used for fluorescence polarization based assay. It is known that the catalytically essential Fe^{II} at the active site of 2-OG oxygenases can be substituted by different transition metals to block the enzyme-catalyzed 2-OG turnover and to avoid the oxidation of Fe^{II} to Fe^{III}.²⁰ In the assay, excess of Mn^{II} was used to PHD2 to ensure that only the metal-bound holo form was present. Additionally, a stable complex was formed by PHD2 with Mn^{II}, 2-OG, and HIF1 α peptide,¹⁹ which indicates that the use of Mn^{II} instead of Fe^{II} has little influence on the binding property of PHD2 protein. Thus, we employ Mn^{II} instead of Fe^{II} as the native metal cofactor to avoid the hydroxylation of the probe FITC-HIF1 α (556–574) while maintaining its binding affinity to PHD2 protein. When the competitive binder exists, the endogenous substrate 2-OG will be displaced from the binding site and the FITC-HIF1 α (556–574) peptide will be released from the complex (Figure 1).²¹ As a consequence, we design a fluorescence polarization based assay using FITC-HIF1 α (556–574) as a probe, which can be used for quantitative and site-specific screening of small molecule PHD2 inhibitors.

Initially, fluorescein-labeled HIF1 α (556–574) peptide was obtained from Shanghai Apeptide Co., Ltd. The binding affinity between the probe (5 nM, Supporting Information (SI) Figure S1) and PHD2 was then examined by fluorescence polarization. It is frustrating that the binding affinity was not strong between FITC-HIF1 α (556–574) peptide and PHD2 in the presence of Mn^{II} (Figure 2). We tried many different methods to optimize

Figure 2. Optimization of the binding conditions for fluorescein-labeled HIF1 α (556–574) (5 nM) and PHD2, binding with PHD2 was measured with additional Mn^{II} and 2-OG, Mn^{II} and no additional 2-OG, and Fe^{II} and NOG in assay buffer.

the binding affinity. Fortunately, when the 2-OG existed, a dramatic increase in fluorescence polarization was observed (Figure 2). This increase indicated significant binding of the relatively small fluorescein-labeled HIF1 α (556–574) peptide to the relatively large PHD2 protein complex when 2-OG existed. This result provides a powerful proof to the design principle of our fluorescence polarization assay. In addition, we have also tried another possible strategy to avoid the hydroxylation of FITC-HIF- α peptide by employing the *N*-oxalyl glycine (NOG), a noncatalytically competent analogue of 2-OG to replace 2-OG, as well as using the native metal ion Fe^{II}. However, as presented in Figure 2, the binding affinity between the probe and PHD2 was greatly decreased in this case. So we chose Mn^{II} and 2-OG as the optimal cofactors. To determine the optimal concentration of 2-OG for our fluorescence polarization assay, different concentrations were determined. As shown in SI Figure S2, we can see that with the declining concentration the binding affinity was gradually reduced. At the concentration of 20 μ M, the binding of PHD2 and FITC-HIF- α probe was saturated. For the optimal binding affinity, we chose the suitable concentration at 20 μ M.

In addition, in order to observe the influence by different transition metal, Zn^{II} and Ni^{II} have also been tested instead of the Mn^{II}. The results showed that there were no significant differences between Mn^{II}, Zn^{II}, and Ni^{II} (Figure 3). Binding

Figure 3. Binding to PHD2 was measured with the addition of Zn^{II} or Ni^{II}, no additional metal, or Fe^{II} at the concentration of 100 μ M in assay buffer.

with PHD2 was also measured with Fe^{II} and no additional metal in the assay buffer, as shown in the Figure 3. When the native Fe^{II} was added to replace Mn^{II}, HIF1 α was catalyzed to hydroxylated peptide as expected, resulting in the dramatic decrease in the signal of fluorescence polarization. When no additional metal was added, the binding affinity was also reduced. These results are all proofs to provide the design principle of our fluorescence polarization assay. In this assay, we used Mn^{II} as the substituted metal to Fe^{II}.

Subsequently, we optimized the concentration of the Mn^{II} (SI Figure S3). We can see that the binding affinity was increased when the concentration reduced to 10 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.

To determine the optimal experimental buffer conditions, we also have studied the dependency of the binding affinity on pH and time. As shown in the SI Figure S4, our experiments indicated that there is virtually no influence of pH on binding properties of PHD2 and fluorescent HIF1 α peptide at pH 6–9; at the pH < 6 or pH > 9, the binding activity of the enzyme was decreased. We thought the cause of this is protein denaturation. Strong acid or alkali buffer will lead to low stabilization of PHD2 and fluorescent HIF1 α peptide, which caused the low binding affinity of the assay. In order to obtain the optimal binding affinity, we chose the physiology pH 7.4 as the suitable pH. We found that the binding of PHD2 and fluorescent HIF1 α reached equilibrium after 1 h of incubation at room temperature, and signals are stable over at least 24 h (SI Figure S5).

We have developed a competition fluorescence polarization assay format in which a test set of PHD2 inhibitors completes with the fluorescent HIF1 α . To elucidate the minimum amount of FITC-HIF1 α that resulted in robust assay, an assay containing various amounts of FITC-HIF1 α without PHD2 was done (SI Figure S1). To avoid the use of high amounts of tracer but also to eliminate possible inconsistencies in concentration measurements, a FITC-HIF1 α concentration of 5 nM appeared optimal for use in competitive assays. The concentration for the PHD2 was 100 nM (Figure 4). At the

Figure 4. Saturation curve of FITC-HIF1 α peptide to PHD2 protein at the optimal experimental buffer conditions.

100 nM PHD2, the obtained assay window of 110 mP is a suitable assay window, which makes the assay sensitive. The assay performance parameters signal-to-noise ratio and Z' were also determined. They are two expressions that have been used to indicate the quality of the assay. As presented in SI Figure 6,

Figure 5. Inhibitory activity of known PHD2 inhibitors against FITC-HIF1 α /PHD2 interaction.

the PHD2 fluorescence polarization assay resulted in excellent S/N (52:23) ratios and Z' values (0.89). The Z' factor is a statistical benchmark to assess the suitability of an assay for high throughput screening (HTS) and is a measure of the reproducibility in the difference in signal between free and bound tracer controls across a large number of assay wells. An assay with ideal reproducibility displays a Z' factor of 1, whereas a Z' factor greater than 0.5 is considered acceptable for a good high-throughput assay.²² The Z' factor calculated for our fluorescence polarization assay is 0.89, confirming that these assay conditions are suitable for HTS.

Dimethyl sulfoxide (DMSO) is a common solvent used in dissolving many compounds used for testing. Thus, the PHD2 fluorescence polarization assay should tolerate the presence of low volumes of DMSO. When we performed the binding and competition assays, a maximum 1.00% DMSO in each well (v/v) was included. We have further tested the effect of up to 16% DMSO on the competition experiments. As presented in SI Figure S7, the assay window is decreased in the presence of DMSO. However, the binding affinity is reasonably stable in the presence of DMSO at least up to 6%.

Fibrogen is one of the pioneers in commercial research on prolyl hydroxylase inhibition and running some of the most advanced stage clinical trials for the therapeutic use of the HIF stabilizers, such as FG-2216 and FG-4592. The second generation FG-4592 (Roxadustat) is currently enrolling for Phase III trials in the USA.^{23,24} Compound 1 was a known inhibitor of PHD2 disclosed in 2007 by the Procter and Gamble Company.²⁵ BAY-85-3934 (Molidustat) is a novel PHD2 inhibitor containing 1,2,3-triazole fragment discovered by Bayer, which is currently enrolling for Phase II trials.²⁶ The inhibitors FG-2216, compound 1, and BAY-85-3934 were studied as competitors due to the fact that the activity of these compounds to PHD2 have been determined by independent methods. To validate that the fluorescence polarization assay results correlate with a relevant biological parameter, we examined the binding affinities of these four PHD2 inhibitors. IC₅₀ values for these four compounds were determined to be as follows: 424.2 ± 1.5 nM for FG-2216, 591.4 ± 13.0 nM for FG-4592, 890.4 ± 5.6 nM for compound 1, and 876.3 ± 11.2 nM for BAY-85-3934 (Figure 5). These results show similar activity values to the reported IC₅₀ of FG-2216 (300 nM),¹⁴ compound 1 (650 nM),²⁵ and BAY-85-3934 (280 nM)²⁷ (Table S1). These results validate the use of this assay to identify small-molecule compounds that disrupt the PHD2-HIF1 α interaction.

In summary, we have established a fluorescence polarization assay for PHD2 using a fluorescent HIF1 α (556–574) peptide as probe. The assays are stable with regard to time and 6%

DMSO and have Z' values ≥ 0.89 , making them well-suited for high-throughput screening.

■ ASSOCIATED CONTENT

■ Supporting Information

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Additional information as noted in the text (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PHD2, prolyl hydroxylase domain 2; 2-OG, 2-oxoglutarate; HIF1 α , hypoxia inducible factor 1 α ; VBC, von Hippel–Lindau protein–Elongin B–Elongin C; FITC, fluorescein isothiocyanate; NOG, *N*-oxalyl glycine; HTS, high throughput screening

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