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Quantitative Analysis of Histone Demethylase Probes Using Fluorescence Polarization

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Abstract

We previously reported methylstat as a selective inhibitor of jumonji C domain-containing histone de-methylases (JHDMs). Herein, we describe the synthesis of a fluorescent analogue of methylstat and its application as a tracer in fluorescence polarization assays. Using this format, we have evaluated the binding affinities of several known JHDM probes, as well as the native cofactor and substrate of JHDM1A. This fluorophore allowed a highly robust and miniaturized competition assay sufficient for high-throughput screening.

INTRODUCTION

Methylation of histone proteins is one of the most important epigenetic modifications.¹ These reversible modifications recruit effector proteins and trigger a wide range of cellular events, including regulation of gene expression, proliferation and differentiation. Histone methylations are closely regulated by histone methyltransferases and histone demethylases. Since 2004, two families of enzymes have been reported to exhibit demethylation activities: FAD-dependent monoamine oxidases (LSD1 and 2) and jumonji C domain-containing histone demethylases (JHDMs).² Compared with LSDs, JHDMs have a much broader substrate scope and can modify lysine residues at all methylation states.

Expression of JHDMs plays critical roles in both development and diseases, such as cancer and mental retardation.³ Overproduction of 2-hydroxy-glutarate, a natural JHDM inhibitor, due to mutation of isocitrate dehydrogenases has been identified in multiple cancers.⁴ This development has led to numerous efforts to develop chemical probes targeting JHDMs. Several classes of α -ketoglutarate (α KG) mimics have been developed to inhibit JHDM

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§Author Contributions

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ASSOCIATED CONTENT

Supporting Information. Experimental details for the syntheses and spectroscopic characterization of the compounds, as well as determination of Z' factor experiment and result in this paper. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

activity,⁵ since all JHDMs use α KG as a cofactor. In addition, a substrate-mimicking small molecule was recently reported to selectively inhibit H3K9-demethylase KIAA1718.⁶ Our group recently discovered a selective, cell-permeable, small-molecule inhibitor methylstat (**1**, Scheme 1), which was designed as a bivalent substrate-cofactor conjugate.⁷ Its corresponding acid, **2** (Scheme 1), selectively inhibits JHDMs in vitro. This bivalent strategy has also proven successful in two very recent reports on JMJD2 class-selective peptidic inhibitors.⁸

Although several classes of JHDM inhibitors have been discovered, determining the selectivity of these inhibitors against various JHDM classes remains a major challenge. This is mainly due to the lack of a uniformed biochemical assay for various JHDM isoforms. Most established JHDM biochemical assays are enzyme inhibition assays.^{5a,9} Due to the self-destructive nature of JHDMs under biochemical reaction conditions,¹⁰ these assays typically require optimization for different JHDM isoforms. In addition, they do not allow for accurate measurement of the dissociation constants of the JHDM probes. Thus, the IC_{50} values derived from these assays cannot be compared directly. Here, we report the synthesis of a fluorescent JHDM probe, **3** (Scheme 1), and the development of a fluorescence polarization (FP)-based binding assay. This assay allows us not only to quantitatively measure the dissociation constants of several JHDM probes, but also to validate the inhibitory mechanism of methylstat.

RESULTS AND DISCUSSION

The synthesis of fluorophore **3** began with conversion of commercially available 4-cyanobenzaldehyde to aldehyde **4** (Scheme 2),¹¹ which then underwent a reductive amination with amine **5**⁷ to afford secondary amine **6**. The *tert*-butoxycarbonyl (Boc) protecting group of **6** was then removed by trifluoroacetic acid (TFA) and ester hydrolyzed under basic conditions. The resulting diamine (**7**) was treated with fluorescein isothiocyanate (FITC) to afford **3** as the major product.

We next evaluated compound **7** and the fluorescent analog **3** in the enzyme inhibition assays against JMJD2A and JMJD3, two of the most sensitive JHDMs to methylstat acid (**2**).⁷ Compound **7** showed half-maximum inhibitory concentrations (IC_{50} s) of 2.4 and 7.3 μ M against JMJD2A and JMJD3, respectively, which are comparable to those of **2** (i.e., 3.3 and 3.1 μ M, respectively). Interestingly, while **3** showed similar activity against JMJD3 with an IC_{50} of 9.5 μ M, it is less active against JMJD2A with an IC_{50} of 23.5 μ M. Furthermore, the IC_{50} values of **3** and **7** against JHDM1A were determined as 0.90 and 0.42 μ M. These results suggest that selective JHDM inhibitors may be obtained by modifying the secondary amine of methylstat.

Compound **3** shows similar fluorescent properties to fluorescein in pH 7.5 aqueous buffer with the maximum excitation and emission wavelengths as 492 and 517 nm, respectively. With the fluorophore in hand, we set out to develop and optimize an FP binding assay to determine the affinity of fluorophore **3** to various JHDMs. Three JHDMs (JHDM1A, JMJD2A and JMJD3) from three different classes were evaluated in a saturation binding experiment by following the protocol reported by Rossi and Taylor.¹² FP signals were recorded using the Envision Multilabel plate reader (Perkin Elmer), and expressed as the change in millipolarization Δ mP, where Δ mP is the mP of the JHDM/**3** mixture minus the mP of **3** only in assay buffer. To determine the dissociation constants (K_d) of **3** and the JHDMs, we plotted JHDM concentrations against Δ mP, and fitted the data using to Equation (1):

$$\Delta mP = [P_{\min} + P_{\max} \times (x/K_d)^n] / [1 + (x/K_d)^n] \quad (\text{Eq. 1})$$

where, $P_{\max}$ and $P_{\min}$ are the maximum and minimum observed ΔmP values, respectively, x is the JHDM concentration, and n is the Hill coefficient of the binding curve. Preliminary tests indicated that **3** bound JHDM1A with the highest affinity among these three JHDMs; therefore, JHDM1A was used for assay optimization.

The initial assay buffer composition was adapted from previous reports on JHDM enzyme activity assays. These generally contain an Fe^{2+} salt and a reducing agent, sodium ascorbate,^{2,7} as Fe^{2+} , but not Fe^{3+} , is a cofactor for JHDM. Our initial results showed that fluorophore **3** binds to JHDM1A with much higher affinity (K_d : 8.6 ± 1.1 nM), compared with JMJD2A and JMJD3, but the $P_{\max}$ was relatively low (< 50 mP, Figure 1A). In the absence of any additional metal ions, the $P_{\max}$ increased significantly (*ca.* 120 mP, Figure 1A); however, the K_d of JHDM1A for **3** also increased significantly (19 ± 1.4 nM, Figure 1A). Taken together, these results suggested the loss of the Fe^{2+} during protein purification and protein degradation in the presence of Fe^{2+} , a common problem for all Fe^{2+} - and αKG -dependent hydroxylases.^{5a} In order to minimize the effects of Fe^{2+} oxidation, the binding assay was evaluated in the presence of Fe^{2+} under anaerobic conditions; the results were similar to those observed under ambient conditions in the presence of ascorbate.

Since JHDM substrate binding necessitates the addition of metal ions, we next examined the binding in the presence of Ni^{2+} or Co^{2+} . Ni^{2+} and Co^{2+} were chosen because they exhibit increased stability under ambient conditions as compared to Fe^{2+} . Ni^{2+} and Co^{2+} have been shown to interact with native substrates as well as inhibitors in the JHDM active site in crystallographic studies; furthermore, these transition metals are capable of inhibiting enzyme activation without altering substrate or cofactor binding in the JHDM active site.¹³ Replacement of Fe^{2+} with Ni^{2+} or Co^{2+} in the assay buffer afforded highly stable binding with a much larger dynamic range (*ca.* 300 mP, Figure 1A). The K_d of JHDM1A for **3** calculated in the presence of Ni^{2+} (9.3 ± 0.5 nM) is more similar to the native condition than that calculated in the presence of Co^{2+} (22 ± 1.4 nM). Hence, we chose to further optimize the FP assay in the presence of Ni^{2+} .

We found the binding of JHDM and **3** reached equilibrium after 4 hours of incubation at room temperature, and the signals are stable over at least 24 hours (Figure S1). The stabilizing effect of the Ni^{2+} buffer on JHDM1A was also observed by SDS-PAGE analysis (Figure S2). JHDM1A in assay buffer containing Fe^{2+} or no additional metal showed significant decomposition after as little as 2 hours, whereas JHDM1A in buffer containing Ni^{2+} showed no observable decomposition over the course of 24 hours. In addition to the divalent metal ion, we have also optimized the buffer and fluorophore concentration. TRIS buffer provided more consistent results than other buffers such as MOPS and HEPES; a stable and reliable FP signal was achieved using 1 nM of fluorophore **3**.

Once the assay conditions were optimized for JHDM1A, we re-evaluated the binding of **3** with JMJD2A and JMJD3. Although both these enzymes bound **3** at high concentrations, saturation of the FP signal was not achieved at up to $10 \mu\text{M}$ protein, the highest concentration tested (Figure 1B). Since a protein fluorophore pair with high binding affinity is required for appropriate resolution of non-fluorescent inhibitors in an FP competition assay, further development of the FP competition assay was performed using only JHDM1A.¹⁴

Next, the ability of **3** to serve as a tracer for FP competitive binding experiments was assessed, and the appropriate concentration of JHDM1A for FP competition assays

optimized. Typically, a protein concentration at which 50–80% of the fluorescent ligand is initially bound is used for FP competition assays, but must be optimized to achieve a desirable dynamic range.^{12,14,15} The competition assay was validated and optimized using pyridine 2,4-dicarboxylic acid (PDCA), a well-known α KG mimic, as the competitive ligand (Figure 2A). The competition assay set up was similar to that of the binding assay except that a constant concentration of protein was used in each condition and the non-fluorescent inhibitor was titrated into the system (5 nM–2 mM). 1 μ L of DMSO (1%) was added to each assay well as a vehicle for the inhibitor. Of the three JHDM1A concentrations tested, 60 nM JHDM1A afforded a large dynamic range appropriate for competition assays (Figure 2A); therefore, 60 nM JHDM1A was selected as the protein concentration for the following FP competition studies.

A series of JHDM probes were evaluated in the optimized FP competition assay, such as methylstat acid (**2**), the JHDM cofactor, α KG, *N*-oxalyl glycine (NOG, an α KG mimic), and the substrate of JHDM1A, an H3K36me2 peptide (Figure 2B).^{5a,13} The results showed that all of the above molecules can displace **3** from JHDM1A. Additionally, the IC₅₀ values stabilized after approximately 4 hours and remained stable for up to 24 hours. These results not only suggest that **3** binds both the substrate and α KG cofactor-binding sites of JHDM1A, but also confirms that methylstat acid (**2**) is a competitive JHDMs inhibitor.

This competition assay also afforded the half inhibitory concentrations (IC₅₀, Figure 2B) of these JHDM-binding molecules. The IC₅₀ values were then used to calculate their dissociation constants (K_i) with JHDM1A using Equation (2) and the online K_i calculator

$$K_i = [I]_{50} / ([L]_{50} / K_d + [P]_0 / K_d + 1) \quad (\text{Eq. 2})$$

where, $[I]_{50}$ and $[L]_{50}$ are free inhibitor concentration and free ligand concentration at 50% inhibition, $[P]_0$ is free protein concentration at 0% inhibition, and K_d is the dissociation constant between protein JHDM1A and **3**.¹⁵

The ability to quantify binding of competitive inhibitors in the JHDM active site is an unprecedented development. Only the binding affinities of some JHDMs relative to histone peptides have been quantified by surface plasmon resonance;^{5a,13c} however, the binding affinity of α KG for various JHDMs has never been reported. Competitive binding assays designed for screening inhibitors have shown relative binding affinities but report IC₅₀ values of competitive inhibitors as opposed to K_i values. Although IC₅₀ values are useful for assessing the relative effectiveness of inhibitors for a single enzyme, they are not directly comparable between enzymes or between different assays, and always depend on the individual assay conditions.¹⁶ Further expansion of our FP binding assay to other JHDM isoforms may be extremely useful for assessing the specificities as well as the inhibitory mechanism of various JHDM probes.

The FP competition assay was further miniaturized to the 384-well plate format. The total volume for each assay was reduced to 20 μ L, while the concentrations of JHDM1A (60 nM), fluorophore **3** (1 nM), and DMSO (1%) remained the same. For high-throughput screening purpose, we also evaluated the influence detergent, Tween 20, to our FP assay. We found that addition of 0.1% Tween 20 to the assay buffer completely abolishes the binding, and 0.001% of Tween 20 is well tolerated. To calculate the Z' factor, we used **2** (100 μ M in DMSO) and DMSO only as positive and negative controls, respectively. Although the dynamic range of this assay decreased slightly (Figure S3), a high Z' factor value (0.78) was calculated,¹⁷ which further demonstrates the robustness of this assay and its suitability for high-throughput screening.

CONCLUSION

We have discovered a fluorescent analogue of methyl-stat, **3**, and have used it to develop an FP binding assay. **3** selectively binds JHDM1A with high affinity (K_d : 9.3 nM) and a large dynamic range (*ca.* 300 mP). Ni^{2+} ion was found not only to be a good surrogate to the native cofactor Fe^{2+} , but it also stabilizes the protein. The binding of **3** to JHDM1A can be displaced by several known JHDM probes, including its cofactor (α KG), substrate (H3K36me2), and methylstat acid (**2**). These results confirm that methylstat acid is a bivalent competitive inhibitor of JHDMS. In addition, this FP competition assay allows quantitative measurement of K_i values of non-fluorescent JHDM1A active site binding molecules. It is also noteworthy that the K_i of α KG and JHDM1A was determined, which has previously been impossible and illustrates the utility of our FP binding assay for quantifying the binding affinities of native JHDM substrates quickly and easily. Furthermore, we were able to use our FP system to develop a highly robust and miniaturized assay appropriate for high-throughput screening of large compound libraries (Z' : 0.78). Further optimization of the fluorophore for the development of FP assays appropriate for other JHDMS is ongoing and will be reported in due course.

EXPERIMENTAL SECTION

Synthetic procedure for the preparation of fluorophore **3**, characterization data, NMR spectra of all new compounds are in supporting information.

Protein expression and purification

Recombinant JHDM1A (1-517) and JMJD3 (1018-1590) were expressed as 6XHis fusion proteins using the pNIC28 and the pNH-TrxT expression vectors, respectively. The coding regions were verified by sequencing and the plasmids were transfected into BL21 *E. coli*. Following expression, JHDM1A was purified using Ni Sepharose™ 6 Fast Flow beads (GE) by gravity chromatography according to the manufacturer's instructions. JMJD3 was purified using cobalt (high density) agarose beads (Gold Biotechnology) according to the manufacturer's protocol. JMJD2A was expressed and purified as described previously.⁷ The purified proteins were exchanged into assay buffer, flash frozen in liquid nitrogen and stored at $-80^{\circ}C$.

FP binding Assay

FP binding experiments were performed in black, low-binding, half-area 96-well plates (Corning 3993). 80 μ L of JHDM1A (2.44 nM–2.50 μ M in two-fold dilution) in assay buffer (50 μ M $NiCl_2$, 25 mM TRIS, 100 mM NaCl, pH 7.5) were added to experimental wells. After 10 minutes, 20 μ L of **3** (5 nM in assay buffer) was then added to each well. Wells containing protein only were subtracted from assay wells as background. Plates were incubated at room temperature and read five times at each time point (1, 2, 4, 6, 8, 10, and 24 h) and the average values at each time point were used for the calculation of the polarization values. Binding curves were fit using KaleidaGraph (v4.1.1, Synergy Software).

FP competitive binding assay

Known JHDM active site binders (**2**, α KG, PDCA, NOG, or peptide H3K36me2) were tested for their ability to compete the binding of **3** to JHDM1A. Two-fold serial dilutions of compounds were prepared as 100x solutions in DMSO. 80 μ L of JHDM1A (75 nM in assay buffer) were added to each well, to which 1 μ L of the above compound solutions were added. The mixtures were incubated at room temperature for 30 minutes prior to addition of 20 μ L of **3** (5 nM in assay buffer). Controls containing **3** and JHDM1A or JHDM1A and the competing compound were included for background subtraction. Each experiment was

performed in duplicate. The assay plates were incubated at room temperature for 4 hours before signals were recorded by the Envision Multilabel plate reader. The calculated data was fitted using KaleidaGraph (v4.1.1, Synergy Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

JHDM	jumonji C domain-containing histone demethylase
αKG	α -ketoglutarate
FP	fluorescence polarization
Boc	<i>tert</i> -butoxycarbonyl
TFA	trifluoroacetic acid
FITC	fluorescein isothiocyanate
PDCA	pyridine 2,4-dicarboxylic acid
NOG	<i>N</i> -oxalyl glycine
K_d	dissociation constant
IC₅₀	half maximum inhibitory concentration
K_i	dissociation constant of inhibitor

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Figure 1.

Optimization of the binding conditions for JHDMs and **3**. A) Binding to JHDM1A was measured with the addition of Fe^{2+} , no additional metal, or Ni^{2+} in assay buffer. B) Binding of **3** to JHDM1A, JMJD2A, and JMJD3 was assessed. Only the binding of JHDM1A and **3** reaches saturation at the concentrations tested.

Figure 2. FP competition assay optimization and results. A) 15, 30, and 60 nM of JHDM1A were tested in the FP competition assay with PDCA. B) The FP competition assay was used to determine IC₅₀ and K_i values for known JHDM probes.

Scheme 1.

Structures of methylstat (1), methylstat acid (2), and its fluorescent analogue methylstat^{fluor} (3)

Scheme 2. Synthesis of fluorophore **3**^a

^aReagents and conditions: (a) LiAlH₄, THF, reflux, 12 h; Boc₂O, Et₃N, THF, 25°C, 12 h; Dess-Martin periodinane, CH₂Cl₂, 25°C, 30 min, 72% for 3 steps; (b) AcOH, NaBH₃CN, MeOH, 25°C, 12 h, 59%; (c) TFA, CH₂Cl₂, 0 °C, 30 min; LiOH, THF, H₂O, 25°C, 2 h, 94% for 2 steps; (d) fluorescein isothiocyanate, sat NaHCO₃/THF, 25 °C, 12 h, 37%.