A Fluorescence Polarization Biophysical Assay for the Naegleria DNA Hydroxylase Tet1
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Supporting Information

ABSTRACT: The discovery of the 5-methylcytosine (5mC) oxidation by the ten−eleven translocation (Tet) protein family was an important advancement in our understanding of DNA-modified epigenetics. Potent inhibitors of these proteins are greatly desired for both the understanding of the functions of these enzymes and to serve as eventual therapeutic leads. So far, the discovery of such small molecules with high affinity has been quite limited. Original tools to screen for activity are greatly needed in order to accelerate this process. Here we present a novel fluorescent probe, and the results of a fluorescence polarization-based binding assay for Naegleria Tet1, a homologue to mammalian Tet. A fluorescence polarization-based competition assay was also established and applied to the rapid and quantitative measurement of the binding affinity of the cofactor αKG and several known Tet1 inhibitors.

KEYWORDS: Ten−eleven translocation (Tet) proteins, fluorescence polarization (FP), epigenetics, 5-hydroxymethylcytosine

The methylation of DNA has long been known as a repressive epigenetic mode of gene regulation. Its importance has been well established in developmental processes, and aberrant patterns have been linked to various pathologies such as cancer. In mammalian cells, the principal modification is at the 5′ position of cytosine and is installed via S-adenosylmethionine (SAM) by various DNA methyltransferases (DNMTs). Investigations into the mechanisms and dynamics of DNA methylation were greatly abetted by the discovery of the ten−eleven translocation (Tet) proteins, which were shown to be capable of the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and eventually to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). These products are then recognized and excised by thymine DNA glycosylase (TDG) and repaired through the base-excision repair (BER) pathway in what is now recognized as an "active" DNA demethylation pathway to restore an unmodified state.

Significant questions remain, however, about these essential proteins and the exact mechanisms of demethylation, as other pathways have been suggested. In the family, all three members (Tet1−3) have been shown capable of oxidizing 5mC, but there is evidence to suggest that these genes play both redundant and distinct roles in different developmental stages and tissues. Elucidation of their unique functions and roles in disease states is also greatly desired. Furthermore, the 5hmC mark has been suggested to be not just an intermediate in the demethylation pathway, but a genuine epigenetic mark in its own right complete with reader proteins.

Currently, methods for detecting Tet activity are quite limited. There have been reports of using ELISA-based commercially available colorimetric kits and a 2D-TLC or mass spectrometry-based procedure to characterize Tet activity in vitro and in vivo. Other methods simply rely on detecting levels of 5hmC via immunoblotting. Clearly, new tools are needed to help expand our understanding of the complexities of DNA-modified epigenetics and the Tet protein family.
techniques of screening for Tet binders would fulfill this need as they are desirable for both research and pharmaceutical purposes. Encouraged by our previous success with the development of fluorescence polarization (FP)-based assays for histone demethylases, we recently developed novel FP-based binding and competition assays for *Naegleria* Tet1 (NgTet1).

FP is uniquely advantageous in studying protein–ligand interactions and has enjoyed wide application in both clinical and biomedical fields. The technique does not require a separation of bound vs free ligand, and so there is no need to perturb that equilibrium during measurements. Furthermore, compared with traditional biochemical enzymatic inhibition assays, FP does not induce changes in either the cofactor or enzyme active site. These FP probes which have been applied to the establishment of FP-based binding and competition assays for the JmC-domain containing histone demethylases JHDM1A and JMJD2A (Figure 1).22,23

NgTet1 is a homologue to mammalian Tet proteins with a simpler domain architecture with 39% similarity.15,28 More importantly, NgTet1 exhibited superior stability to mTet1. The purified NgTet1 was used to screen readily available collection of fluorescent probes in saturation binding experiments.24 These fluorescent probes each contains an α-ketoglutarate (αKG)-mimicking fragment, which presumably binds to the enzyme active site.

Saturation binding experiments were performed as previously described.22 The FP signals were recorded on an Envision Multilabel plate reader (PerkinElmer) and expressed as change in millipolarization, ∆mP, comparing the signal of fluorophore in buffer alone to the signal of fluorophore and protein. Concentration of NgTet1 was plotted versus ∆mP, and the data were fit to the following equation using KaleidaGraph (v4.1.1, Synergy Software) to determine the $K_d$ of fluorescent tracer:

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

where $P_{\text{min}}$ and $P_{\text{max}}$ are the minimum and maximum observed ∆mP values, $x$ is the concentration of NgTet1, and $n$ is the Hill coefficient of the binding curve.

A novel fluorescent probe 3 (Figure 2A) was found to display the strongest binding to NgTet1, among 12 fluorophores evaluated. Optimization of the assay buffer was next conducted with the addition or absence of a variety of metal ions (Fe$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, or none) to determine the best conditions to preserve the stability of the protein. In agreement with previous results, we determined that addition of 50 μM of NiCl$_2$ was optimal as nickel exhibits increased stability to oxidation under ambient conditions. Signals were observed to stabilize after 4 h and lasted up to 20 h, which is also consistent with previously developed FP assays.22,23 The $K_d$ was calculated to be 250 ± 36 nM (Figure 2B).

With the working fluorescent tracer in hand, we next sought to develop a FP-based competition assay in order to assess the binding affinity of several known probes of the Tet proteins. The concentration of NgTet1 used for these experiments was two times the $K_d$ of the fluorophore (500 nM) as it offered a suitable dynamic range in the competition assay. The nonfluorescent small molecule to be tested was titrated against a constant concentration of NgTet1 and 3 at a starting concentration of 2500 or 5000 μM in 2-fold serial dilutions. The FP signals were recorded as before, and the concentration of inhibitor was plotted versus ∆mP and similarly fit with eq 1 to afford the half-maximum inhibitory values in place of the fluorophore $K_d$. These IC$_{50}$s were then used to calculate the dissociation constants of the inhibitors ($K_i$) using eq 2:

$$
K_i = [I]_{50} / ([L]_{50} / K_d + [P]_{50} / K_d + 1)
$$

where $[I]_{50}$ and $[L]_{50}$ are the free inhibitor and free ligand concentration at 50% inhibition, $[P]_{50}$ is the free protein concentration at 0% inhibition, and $K_d$ is the dissociation constant between NgTet1 and 3. The results were summarized in Table 1.

![Figure 1. Structures of two previously discovered fluorescent probes of αKG- and Fe$^{2+}$-dependent hydroxylases JHDM1A and JMJD2A, respectively.](image)

![Figure 2. Discovery of a fluorescent probe 3 for NgTet1. (A) Structure of fluorescent probe 3. (B) Binding curve of NgTet1 and 3 under optimized conditions.](image)
Among the six compounds tested, only three showed the ability to competitively displace 3 at the concentrations tested: the cofactor, αKG (4), and two structural analogues, N-oxalyl glycine (NOG, 5), and 2,4-pyridinedicarboxylic acid (PDCA, 6). Both NOG and PDCA are known inhibitors of many αKG-dependent dioxygenases. The IC$_{50}$s were found to be 250, 49, and 27 μM, and the $K_i$s were calculated to be 83, 16, and 9.0 μM, respectively. Their competitive binding curves are visualized in Figure 3. The lower binding affinity of αKG compared with its structural analogues is unexpected but may suggest that a conformational difference exists between this purified protein and its complex with the DNA substrate.

D-2-Hydroxyglutaric acid (D-2-HG, 7) is also structurally similar to αKG, differing only with the replacement of a hydroxyl in place of the ketone at the C-2 position. This compound has been shown to be weakly competitive with αKG with both histone demethylases and the Tet family in the millimolar range. \(^{26}\) Indeed, very slight inhibition was observed at the highest concentrations tested (2.5 and 1.25 mM, Figure 3); however, complete or even 50% displacement of 3 was not observed. An IC$_{50}$ of 5.2 mM was obtained through fitting the data in KaleidaGraph, and its $K_i$ was calculated to be 1.7 mM.

The results presented here show that the FP-based binding and competition assays allow the quantification and comparison of binding affinities of small-molecule inhibitors to the active site of NgTet1. The novel fluorescent probe 3 displays 250 nM binding affinity that allows for a wide resolution of inhibitor potency. \(^{27}\) αKG and two analogues, NOG and PDCA, were shown to competitively displace 3 with low micromolar affinity to NgTet1. A Z’ factor of 0.73 (SI Figure S1) was calculated with PDCA and DMSO as positive and negative controls, respectively, which demonstrates the reproducibility of this assay. Miniaturization of the competition assay will provide a useful technique for the high-throughput screening of large compound libraries to identify novel inhibitors of Tet proteins. Future work entails testing the potency of the fluorescent probes against much larger mammalian Tet protein and the identification of additional chemical probes that preferentially bind to each member of the Tet family, as this would allow direct comparison of specificity between each member.

### EXPERIMENTAL PROCEDURES

**FP Binding Assay.** Experiments were performed in duplicate in black, low-binding half area 96-well plates (Corning 3993). First, 80 μL of NgTet1 (4 μM to 4 nM final concentrations in 2-fold serial dilutions) in assay buffer (25 mM TRIS, 100 mM NaCl, 50 μM NiCl$_2$, pH 7.0) were added to the experimental wells. Then 10 μL of 3 (1 nM final concentration in assay buffer) were added to the wells. Wells containing only protein were subtracted as background. The wavelengths for excitation and emission were 492 and 517 nM, respectively. The plate was incubated at room temperature for 4 h, and then the FP signals were recorded three times and averaged.

**FP Competitive Binding Assay.** Experiments were performed in duplicate in black, low-binding half area 96-well plates (Corning 3993). Two-fold serial dilutions of the compounds 4–9 were prepared as 100× solutions in DMSO. First, 80 μL of NgTet1 (500 nM final concentration) in assay buffer (25 mM TRIS, 100 mM NaCl, 50 μM Na$_2$EDTA) was added to each well. Then 5 μL of 25 mM DMSO (for negative controls) or the tested compounds was added to the wells. The plate was incubated at room temperature for 4 h, and then the FP signals were recorded three times and averaged.
NiCl₂ (pH 7.0) was added to the experimental wells. Then, 1 μL of the compound solutions was added and incubated for 20 min prior to the addition of 20 μL of 1 nM final concentration in assay buffer. Controls with only 3 and inhibitor or only 3 and protein were used for background subtraction and to calculate maximum and minimum binding of 3 to the protein. The plate was incubated at room temperature for 4 h, and then the FP signals were recorded three times and averaged.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmедчhemlett.5b00366.

Protein purification, determination of the Z' factor, and an image of the purified protein (PDF)

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**Author Contributions**

These authors contributed equally to this work. L.J.M. developed and ran the FP assays; W.W. purified the protein; L.J.M., Y.Z., and X.W. prepared the manuscript. All authors have given approval to the final version of the manuscript.

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### Notes

The authors declare no competing financial interest.

### ABBREVIATIONS

Tet protein, ten—eleven translocation protein; FP, fluorescence polarization; rKG, alpha-ketoglutarate; NOG, N-oxalyl glycine; D-2-HG, d-2-hydroxyglutaric acid; PDCA, pyridine 2,4-dicarboxylic acid; Kᵦ, dissociation constant; IC₅₀, half-maximum inhibitory concentration; Kₑ, dissociation constant of inhibitor

### REFERENCES

(1) Bird, A. DNA methylation patterns and epigenetic memory. *Genes Dev.* 2002, 16, 6–21.

(2) Küllis, M.; Esteller, M. DNA methylation and cancer. *Adv. Genet.* 2010, 70, 27–56.

(3) Scouriz, L.; Mouly, E.; Bernard, O. A. Tet proteins and the control of cytosine demethylation in cancer. *Genome Med.* 2015, 7, 9.

(4) Okano, M.; Xie, S.; Li. E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat. Genet.* 1998, 19, 219–220.

(5) Goll, M. G.; Bestor, T. H. Eukaryotic cytosine methyltransferases. *Annu. Rev. Biochem.* 2005, 74, 481–514.

(6) Tahiliani, M.; Koh, K. P.; Shen, Y.; Pastor, W. A.; Bandukwala, H.; Brudno, Y.; Agarwal, S.; Iyer, L. M.; Liu, D. R.; Aravind, L.; Rao, A. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 2009, 324, 930–935.

(7) Ito, S.; Shen, L.; Dai, Q.; Wu, S. C.; Collins, L. B.; Swenbgberg, J. A.; He, C.; Zhang, Y. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylysine. *Science* 2011, 333, 1300–1303.

(8) Lu, X.; Zhao, B. S.; He. C. TET family proteins: oxidation activity, interacting molecules, and functions in disease. *Chem. Rev.* 2015, 115, 2225–2239.

(9) He, Y. F.; Li, B. Z.; Li, Z.; Liu, P.; Wang, Y.; Tang, Q.; Ding, J.; Jia, Y.; Chen, Z.; Li, L.; Sun, Y.; Li, X.; Dai, Q.; Song, C. X.; Zhang, K.; He, C.; Xu, G. L. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* 2011, 333 (6047), 1303–1307.

(10) Zhao, H.; Chen, T. Tet family of 5-methylcytosine dioxygenases in mammalian development. *J. Hum. Genet.* 2013, 58 (7), 421–427.

(11) Wu, H.; Zhang, Y. Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. *Genes Dev.* 2011, 25, 2436–2452.

(12) Piccolo, F. M.; Bagci, H.; Brown, K. E.; Landeira, D.; Soza-Ried, J.; Feytout, A.; Mooijman, D.; Hajkova, P.; Leitch, H. G.; Tada, T.; Kriaucionis, S.; Dawlaty, M. M.; Jaenisch, R.; Merkenschlager, M.; Fischer, A. G. Different roles for Tete1 and Tete2 proteins in reprogramming-mediated erasure of imprints induced by EGC fusion. *PLoS Genet.* 2013, 9 (6), 1003396.

(13) L.; He, X.; Jin, S.; Iqbal, K.; Shi, Y.; Deng, Z.; Szabo, P. E.; Pfeifer, G. P.; Li, J.; Xu, G. The role of Tete DNA dioxygenase in epigenetic reprogramming by oocytes. *Nature* 2011, 477, 606–610.

(14) Huang, Y.; Rao, A. Connections between TET proteins and aberrant DNA modification in cancer. *Trends Genet.* 2014, 30 (10), 464–474.

(15) Tan, L.; Shi, Y. G. Tet family proteins and 5-hydroxymethylcytosine in development and disease. *Development* 2012, 139, 1895–1902.

(16) Spruijt, C. G.; Gnerlich, F.; Smits, A. H.; Pfaffeneder, T.; Jansen, P. W. T. C.; Bauer, C.; Münzel, M.; Wagner, M.; Müller, M.; Khan, F.; Eberl, H. C.; Mensinga, A.; Brinkman, A. B.; Leplikov, K.; Müller, U.; Walter, J.; Boelens, R.; van Ingen, H.; Leonhardt, H.; Carell, T.; Vermeulen, M. Dynamic readers for 5-(hydroxyl)methylcytosine and its oxidized derivatives. *Cell* 2013, 152 (5), 1146–1159.

(17) Véron, N. Tet proteins: on track towards DNA demethylation? *Biomol. Concepts* 2012, 3, 395–402.

(18) Shen, L.; Zhang, Y. 5-Hydroxymethylcytosine: generation, fate, and genomic distribution. *Curr. Opin. Cell Biol.* 2013, 25 (3), 289–296.

(19) Bachman, M.; Uribe-Lewis, S.; Yang, X.; Williams, M.; Murrell, A.; Balasubramanian, S. 5-Hydroxymethylcytosine is a predominantly stable DNA modification. *Nat. Chem.* 2015, 6 (12), 1049–1055.

(20) Nettersheim, D.; Heukamp, L. C.; Fronhoffs, F.; Grewe, M. J.; Haas, N.; Waha, A.; Honecker, F.; Waha, A.; Kristiansen, G.; Schorle, H. Analysis of TET expression/activity and Smc oxidation during normal and malignant germ cell development. *PLoS One* 2013, 8 (12), e82881.

(21) Shen, L.; Zhang, Y. Enzymatic analysis of Tet proteins: key enzymes in the metabolism of DNA methylation. *Methods Enzymol.* 2012, 512, 93–105.

(22) Xu, W.; Podoll, J. D.; Dong, X.; Tumber, A.; Oppermann, U.; Wang, X. Quantitative analysis of histone demethylase probes using fluorescence polarization. *J. Med. Chem.* 2013, 56, 5198–5202.

(23) Wang, W.; Marholz, L. J.; Wang, X. Novel scaffolds of cell-active histone demethylase inhibitors identified from high-throughput screening. *J. Biomol. Screening* 2015, 20 (6), 821–827.

(24) Xu, W.; Wang, X. Histone demethylase inhibitors and methods for using the same. U.S. Patent 20130137720 A1, May 20, 2013.

(25) Nikolovska-Coleska, Z.; Wang, R.; Fang, X.; Pan, H.; Tomita, Y.; Li, P.; Roller, P. P.; Krajewski, A.; Saito, N. G.; Stuckey, J. A.; Wang, S. Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. *Anal. Biochem.* 2004, 326, 261–273.

(26) Xu, W.; Yang, H.; Liu, Y.; Yang, Y.; Wang, P.; Kim, S.; Ito, S.; Yang, C.; Wang, P.; Xiao, M.; Liu, L.; Jiang, W.; Liu, J.; Zhang, J.; Wang, B.; Frye, S.; Zhang, Y.; Xu, Y.; Lei, Q.; Guan, K.; Zhao, S.; Xiong, Y. Oncometabolite 2-hydroxylactic acid is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases. *Cancer Cell* 2011, 19 (1), 17–30.
(27) Huang, X. Fluorescence polarization competition assay: the range of resolvable inhibitor potency is limited by the affinity of the fluorescent ligand. *J. Biomol. Screening* **2003**, *8*, 34−38.
(28) Hashimoto, H.; Pais, J. E.; Zhang, X.; Saleh, L.; Fu, Z. Q.; Dai, N.; Correa, I. R., Jr.; Zheng, Y.; Cheng, X. Structure of a Naegleria Tet-like dioxygenase in complex with 5-methylcytosine DNA. *Nature* **2014**, *506* (7488), 391−395.