JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
Cite This: J. Am. Chem. Soc. 2018, 140, 10263−10269
pubs.acs.org/JACS

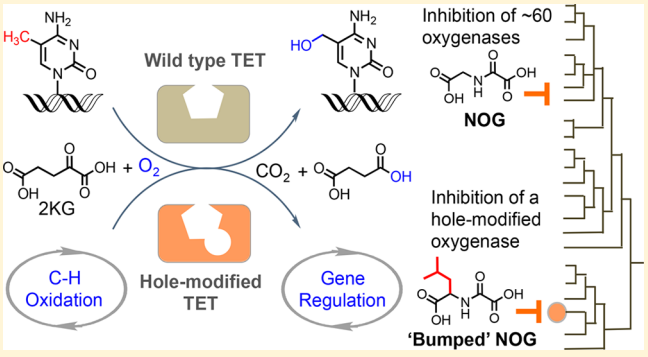
Complementary Steric Engineering at the Protein−Ligand Interface for Analogue-Sensitive TET Oxygenases

Babu Sudhamalla, Sinan Wang, Valerie Snyder,[§] Sam Kavoosi,[§] Simran Arora,[§] and Kabirul Islam*

Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, United States

Supporting Information

ABSTRACT: Ten-eleven translocation (TET) enzymes employ O_2, earth-abundant iron, and 2-ketoglutarate (2KG) to perform iterative C−H oxidation of 5-methylcytosine in DNA to control expression of the mammalian genome. Given that more than 60 such C−H oxygenases are present in humans, determining context-dependent functions of each of these enzymes is a pivotal challenge. In an effort to tackle the problem, we developed analogue-sensitive TET enzymes to perturb the activity of a specific member. We rationally engineered the TET2−2KG interface to develop TET2 variants with an expanded active site that can be specifically inhibited by the *N*-oxalylglycine (NOG) derivatives carrying a complementary steric "bump". Herein, we describe the identification and engineering of a bulky gatekeeper residue for TET proteins, characterize the orthogonal mutant−inhibitor pairs, and show generality of the approach. Employing cell-permeable NOG analogues, we show that the TET2 mutant can be specifically inhibited to conditionally modulate cytosine methylation in chromosomal DNA in intact human cells. Finally, we demonstrate application of the orthogonal mutant−inhibitor pair to probe transcriptional activity of a specific TET member in cells. Our work provides a general platform for developing analogue-sensitive 2KG-dependent oxygenases to unravel their functions in diverse signaling processes.



INTRODUCTION

Organismal multicellularity has been mechanistically linked to a steady increase in oxygen pressure in the atmosphere.[1] This is likely due to the emergence of enzymes catalyzing oxygen-dependent biochemical reactions essential to metabolic and transcriptional regulations. An early class of such enzymes employ simple co-substrates such as 2-ketoglutaric acid (2KG) **1** and iron for oxidative processing of proteins and nucleic acids.[2] In humans, more than 60 such oxygenases catalyze C−H hydroxylation in diverse nuclear and cytoplasmic substrates and contribute significantly to cellular processes ranging from DNA repair to hypoxic signaling.[2] Within this group, ten-eleven translocation (TET) enzymes perform C−H oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) to control chromatin accessibility and thereby expression potential of the human genome (Figure 1).[3,4]

TET-mediated successive oxidation of 5mC (once thought to be a permanent mark because of the stable C−C connectivity) to 5hmC, 5-formylcytosine (5fC), and finally 5-carboxylcytosine (5caC) provides a biochemical basis for active DNA demethylation.[5] 5caC could be recognized and excised by thymine DNA glycosylase (TDG) to effect base excision repair (BER)-mediated replacement of 5caC with deoxycytidine (dC).[6] Direct decarboxylation of 5caC could be another potential mechanism of active genome demethylation, although more evidence is needed.[7,8] Gene regulation by such oxidative demethylation is critical for cellular differentiation and embryogenesis. For example, TET-mediated demethyla-

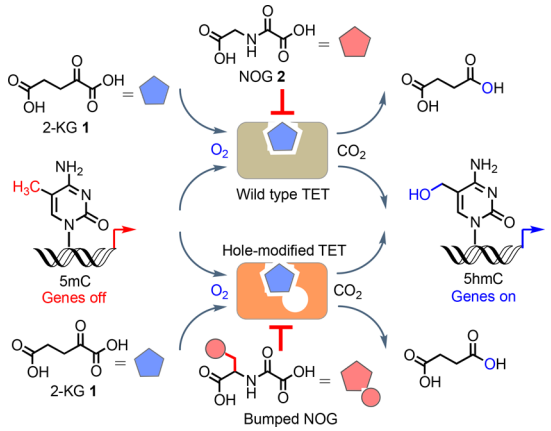


Figure 1. Logic of analogue-sensitive (*as*) TET enzymes. Conserved bulky amino acids are replaced to introduce hole-modified silent mutations in the active site. The mutants, although acting like wild-type TETs, could be specifically inhibited by "bumped" *N*-oxalylglycine (NOG) analogues that do not perturb native 2KG-dependent enzymes.

Received: May 20, 2018
Published: July 20, 2018

DOI: 10.1021/jacs.8b05283
J. Am. Chem. Soc. 2018, 140, 10263−10269

tion on the paternal chromosome is essential to early development of a fertilized egg.[9] Aberrant gene regulation by TET overexpression and somatic mutation are implicated in multiple human conditions.[10] Interrogating developmentally essential and clinically relevant TET members with distinct as well as compensatory functions in fluctuating cellular environments constitutes a major challenge.[11]

Intriguing chemical transformations (iterative C−H functionalization under ambient conditions) coupled with asymmetric demethylation (paternal over maternal pronuclei) and the causal role in human disease have generated widespread interest in studying how a specific TET protein reprograms our genome in diverse physiological contexts.[12] Diagramming a TET-specific gene regulatory network with genetic perturbation is difficult owing to its slow acting nature and oftentimes loss of the entire protein. Pharmacological perturbation involving small molecules provides a powerful alternative to dissect protein functions with high temporal control.[13,14] However, attempts to develop small-molecule probes for TET enzymes with cellular activity have not met with much success as of yet.[15] Gaining specificity from such molecules would be challenging due to the similarity in active site fold, cofactors, and co-substrates among 60 2KG-dependent human oxygenases.

To address these challenges, we envisioned developing orthogonal mutant−inhibitor pairs of TET enzymes by engineering a steric component of the TET−2KG interface. In this approach, known as analogue-sensitive chemical genetics or the bump-and-hole tactic,[16−23] the active site of TET enzymes is modified by replacing bulky residues with alanine or glycine (Figure 1). The catalytically competent TET variants with expanded active site are then screened for selective inhibition by 2KG-competitve molecules. The mutant-specific inhibitors are obtained from *N*-oxalylglycine (NOG) **2**, a nonspecific inhibitor of 2KG-utilizing enzymes,[24] by appending complementary steric bulk at a suitable position (Figure 1). Some of these NOG analogues are expected to be refractory to the wild-type enzymes due to a potential steric clash with the larger amino acids in the active site, thus providing chemical probes to interrogate a specific TET member. We selected NOG as the inhibitor scaffold because of its ability to bind the TET enzymes in a 2KG-competitive manner due to structural similarity.[25,26] The presence of an amido acid functionality in NOG (in place of the ketoacid in 2KG) prevents its catalytic processing by the enzymes, thus making it an effective inhibitor. In this study, we describe our efforts toward development of the first analogue-sensitive TET−NOG pairs and their application in probing transcriptional activity of a specific TET member in human cells.

RESULTS AND DISCUSSION

Development of the Orthogonal TET2−NOG Pair. We selected TET2 as a representative DNA demethylase here because of its pivotal role in global methyl erasure during embryogenesis, its established link to cancer, and the availability of critical structural information.[25] In order to develop TET2 variants with an expanded active site, we analyzed the crystal structure of TET2 bound with NOG and 5mC-containing DNA and identified several bulky residues that line up to hold 2KG in the active site (Figure 2a). Given that the majority of these amino acids are hydrophobic in nature and do not participate in strong electrostatic interactions, either with 2KG or DNA, we performed alanine

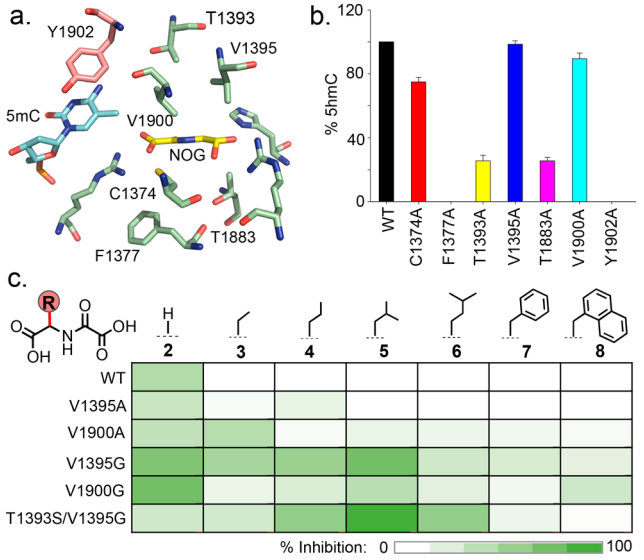


Figure 2. Development of analogue-sensitive (*as*) TET enzymes. (a) Structure of TET2 bound with 5mC-DNA and NOG (PDB: 4NM6) showing conserved bulky hydrophobic amino acids. The selected residues are targeted for mutagenesis. (b) Bar diagram showing activity of selected alanine mutants of TET2 (% 5hmC formation) as judged by MALDI MS analysis. (c) Heat-map representation of % inhibition of selected hole-modified TET2 mutants by a set of NOG analogues. The best mutant T1393S/V1395G is referred to as *as* TET2.

scanning on these sites, one at a time. Mutant proteins carrying the TET2 catalytic domain were expressed and subjected to a MALDI-based C−H hydroxylation assay[26] using a 5mC-containing short DNA and the cofactor 2KG **1** (Figure 2b). The wild-type TET2 and most of the mutants were capable of accepting **1** as their cofactor to generate 5hmC DNA albeit at varying degrees (Figures 2b, S1). F1377A and Y1902A mutants were however completely inert, likely due to the combined effect of active site misfolding and compromised DNA binding. Loss of H-bonding between 5mC and Y1902 may contribute to the catalytic inability of the Y1902A mutant.[25]

This screening data provided us with a platform suitable for developing an analogue-sensitive TET2 enzyme, reminiscent of the protein kinase mutants where engineering a bulky gatekeeper residue sensitized the mutants toward "bumped" inhibitors.[27,28] To obtain such allele-specific inhibitors, we synthesized a series of NOG analogues, **3**−**8**, bearing alkyl and aryl moieties with variegated steric bulk at C4 of NOG **2** (Figure 2c, Schemes S1−S3).[24] Next, we examined the inhibitory activity of the NOG analogues toward TET2 and its mutants using the MALDI-based hydroxylation assay (Figures 2c, S2, S3). Wild-type TET2 and the majority of its mutants were inhibited by NOG **2**, reflecting their ability to bind and process 2KG **1** as cofactor. However, with the exception of the V1900A and V1395A mutants, which were moderately inhibited by the ethyl and propyl NOGs **3** and **4**, respectively, the majority of the mutants remained inert toward the bulky analogues (Figures 2c, S4).

Careful analysis of the NOG-bound TET2 structure revealed that the isopropyl side chains of V1395 and V1900 reside very close (within 4.8 Å) to the C4 methylene group carrying the bulky substituents in NOG analogues. This led us to generate the V1395G and V1900G mutants with a slightly larger active

DOI: 10.1021/jacs.8b05283
J. Am. Chem. Soc. 2018, 140, 10263−10269

site with intact demethylase activity (Figure S5). In the demethylation assay, multiple NOG analogues inhibited the V1395G mutant albeit to varying degrees (Figures 2c, S6). The smaller NOGs such as **4** and **5** inhibited the mutant more effectively. The analogues did not inhibit the V1900G mutant to a significant degree (Figures 2c, S7). Given that removing the steric block at V1395 site sensitizes TET2 mutants toward multiple inhibitors, we reasoned that expanding the active site further might help in gaining stronger inhibition by the bulkier NOGs. We therefore introduced a second site mutation to V1395G to generate multiple double mutants. The majority of the mutants lost their catalytic activity toward 5mC DNA. Remarkably, the T1393S/V1395G mutant retained its DNA demethylase activity as demonstrated by the hydroxylation assay (Figure S5). Furthermore, the double mutant was strongly blocked by NOG analogues **4**–**6** (Figures 2c, S8). Leucine NOG **5** appeared to be particularly effective against the mutant, demonstrating the importance of complementary steric engineering to develop analogue-sensitive (*as*) TET2.

Characterization of the Orthogonal TET2–NOG Pair. We next sought to assess the inhibitory efficacy of NOG analogues (**4**–**6**) by determining their IC_{50} values against the selected TET2 variants. The MALDI-based hydroxylation assay is suitable for providing quantitative information on TET enzymatic activity because of the similar ionization potentials of 5mC- and 5hmC-containing DNAs.[26] The propyl NOG **4** showed a better IC_{50} against the double mutant T1393S/V1395G (hereafter *as* TET2) compared to V1395G (120 vs 473 μM), consolidating the importance of the expanded TET2 active site for inhibitor potency of NOG analogues (Figure S9–S11). From our dose–response experiments, we found leucine NOG **5** to be the most effective inhibitor for *as* TET2, with an IC_{50} of 18 μM (Figures 3a, S12, S13). This compound did not show any inhibition toward wild-type TET2 in a wide range of concentrations (Figures 3a, S14), thus making it suitable for allele-specific perturbation of TET2.

To rationalize the binding potency and specificity of **5** to *as* TET2, we performed docking experiments using AutoDock energy minimization software. For this purpose, we used the coordinates from the NOG-bound TET2 crystal structure (PDB 4NM6).[25] In the most stable conformation, **5** occupied the site of NOG **2** and maintained interactions with the *as* mutant in a manner similar to that of **2** with wild-type TET2 (Figure S15). Most importantly, in the energy-minimized model, the isobutyl side chain of **5** occupied the hole created by V1395G mutation in the *as* variant and induced a steric clash at the active site of the wild-type protein, which explains the observed specificity (Figure 3b,c). We further noticed that the S1393 residue in the mutant makes an extra H-bonding contact with the amido acid group in **5**, potentially contributing to the improved inhibitor efficacy against *as* TET2 (Figure 3d). Overall, the docking study suggests that V1395G and T1393S mutations in *as* TET2 may contribute differentially to the analogue sensitivity, with the former bringing more steric complementarity and the latter more electrostatic affinity.

To further ensure that the "bumped" NOGs do not inhibit other human 2KG-dependent enzymes, we examined a set of structurally and functionally distinct demethylases: TET3 (DNA demethylase), FTO and ALKBH5 (RNA demethylases), KDM4A, C and KDM6B (histone demethylases).[29−35] These wild-type proteins efficiently acted on their respective substrates in the presence of cofactor **1** and were inhibited by

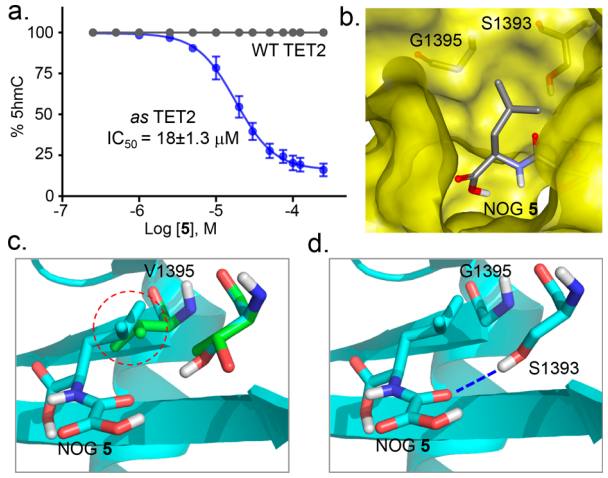


Figure 3. Potency and specificity of the orthogonal pairs. (a) NOG analogue **5** inhibits the steady-state activity of the *as* TET2 variant with an IC_{50} of 18 μM as determined by MALDI MS. The compound did not inhibit wild-type TET2. (b) Energy-minimized structure showing expanded active site pocket in double mutant (T1393S/V1395G) housing the bulky isobutyl group of NOG **5**. (c) Superimposed structures of WT-TET2 and *as* TET2 show steric clash (indicated by the red circle) between the isobutyl group of NOG **5** and the isopropyl side chain of the V1395 residue of WT-TET2. (d) G1395 of *as* TET2 releases the steric clash with NOG **5**. Dashed blue line shows a unique H-bonding contact between the −OH group of S1393 of *as* TET2 and the amido acid moiety of NOG **5** in the energy-minimized structure.

canonical NOG **2** but not the "bumped" NOG analogue **5** (Figures S16–S22). The fact that all seven native demethylases, including TET2, examined thus far remained refractory toward **5** lends further support for the orthogonality of the inhibitor across human 2KG-dependent enzymes.

Next, we examined the activity of the mutant–inhibitor pair on complex substrates (Figure 4a). We collected genomic DNA from human embryonic kidney 293T (HEK293T) cells. In a dot-blot assay, using the appropriate antibody, the isolated genomic DNA showed a robust level of methylated cytidine (5mC) and an almost undetectable amount of 5hmC, consistent with minimal expression of the TET enzymes in these cells (Figure 4b).[36,37] Hydroquinone treatment, however, induced 5hmC formation in the genomic DNA of cultured cells, likely via activation of the endogenous TET enzymes as reported earlier (Figure S23).[38] Upon incubating the genomic DNA with wild-type and *as* TET2 along with the cofactor **1**, we observed significant formation of 5hmC (Figure 4b,c,d) in both cases, suggesting that the hole-modified mutant is capable of acting on a complex substrate with efficiency comparable to that of the wild-type protein. More importantly, leucine NOG **5** completely abrogated the formation of 5hmC by *as* TET2 but not the wild-type enzyme, which was inhibited only by NOG **2**. Collectively, these results demonstrate that the mutant–inhibitor pair is truly orthogonal and functional on a physiologically relevant substrate.

Development of Analogue-Sensitive TET1 and TET3 Variants. After demonstrating that *as* variant can be successfully developed for TET2, we sought to expand the technology to other members of the family. In mammals, the TET family consists of three members, TET1–3, each with distinct functions.[7] Sequence analyses of TET oxygenases revealed that the amino acids corresponding to T1393 and

DOI: 10.1021/jacs.8b05283
J. Am. Chem. Soc. 2018, 140, 10263−10269

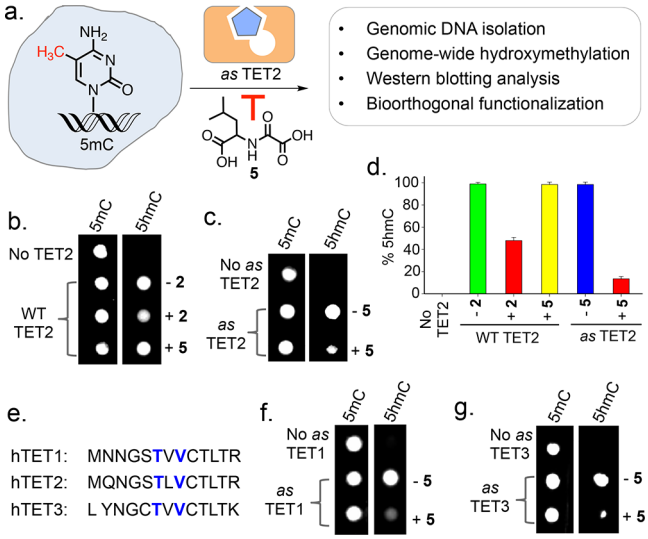


Figure 4. Activity of *as* TET2–NOG **5** pairs on the genomic DNA. (a) Schematic showing isolation of genomic DNA from human cells followed by enzymatic hydroxylation of 5mC by *as* TET2 and its inhibition by **5** and subsequent biochemical analysis. (b) Dot-blot assay showing activity of wild-type TET2 on the genomic DNA. The activity was inhibited by NOG **2** but not by "bumped" NOG **5**. (c) Dot-blot assay showing activity of *as* TET2 on the genomic DNA, which was inhibited by the "bumped" NOG **5**. (d) Quantitative representation of the dot-blot results obtained in (b) and (c). (e) Sequencing data showing T1393 and V1395 of TET2 are fully conserved among other TET members. (f, g) Dot-blot assays showing activity of *as* TET1 and TET3, respectively, on the genomic DNA and their inhibition by NOG **5**.

V1395 in TET2 are highly conserved among TET1–3 (Figure 4e).[29] These bulky amino acids are very likely acting as gatekeeper residues that preclude binding of bulky NOG analogues as observed for TET2. We generated T1683S/V1685G and T1088S/V1090G double mutants of TET1 and -3, respectively, as their *as* variants. When examined for activity, the TET1 and -3 mutants efficiently produced 5hmC in genomic DNA as measured in the dot-blot assay (Figure 4f,g), confirming their catalytic activity. We subsequently showed that the *as* variants were selectively inhibited by NOG analogue **5** (Figure 4f,g). Collectively, our results provide strong evidence for the existence of a bulky gatekeeper residue (an equivalent of V1395 in TET2) within the TET subfamily. Mutations at these sites led to, for the first time, analogue-sensitive DNA demethylases, suitable to deconvolute their specific functions. We have shown earlier that hole-modifying F185G mutation of KDM4A, a 2KG-dependent histone demethylase, sensitizes the mutant toward a series of bulky 2KG cofactor analogues.[23] F185 and V1395 residues in KDM4A and TET2, respectively, reside in close proximity to the cofactor 2KG in the respective active sites. These amino acids prevent binding of the "bumped" cofactor/inhibitor analogues to the wild-type proteins by acting as gatekeeper residues. Our studies disclose a potentially general design principle toward engineering these gatekeeper sites to achieve analogue sensitivity for the 2KG-dependent human enzymes.

Functionalization of TET-Specific Activity on Genomic DNA. Chemoenzymatic functionalization of 5hmC followed by affinity enrichment and sequencing has become a powerful method to map the genomic distribution of cytosine modifications and hence TET activity.[39] A particular approach includes β-glucosyltransferase (β-GT)-mediated glycosylation of 5hmC present in genomic DNA with 6-azidoglucose-UDP followed by "click" ligation with the appropriate reporter alkyne.[39,40] We sought to combine the *as* TET variants with the chemoenzymatic modification of 5hmC that would potentially allow direct probing of genome-wide activity of a specific TET member (Figure 5a). 5hmC generated on a short

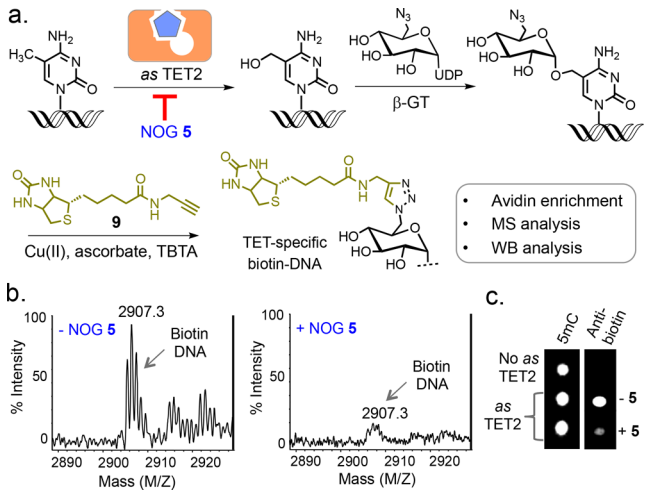


Figure 5. Chemoenzymatic labeling of genome-wide TET activity. (a) Schematic showing steps in the β-GT/"click" chemistry mediated biotinylation of 5hmC generated by *as* TET2 on genomic DNA. (b) MALDI MS showing successful azido-glucosylated and "clicked" product only in the absence of **5**. (c) Dot-blot assay with antibiotin antibody confirmed tandem azido-glucosylation and biotinylation of 5hmC on genomic DNA. NOG analogue **5** strongly inhibited such derivatization.

DNA substrate by the *as* TET2 mutant underwent smooth glycosylation to 6-azidoglucose-UDP using β-GT followed by Cu-catalyzed "click" ligation[41] with biotin-alkyne **9**, as confirmed by MALDI MS (Figures 5b, S24, Scheme S4). However, no such ligated product was obtained when the TET2 assay was performed in the presence of "bumped" NOG **5** (Figure 5b), thus correlating the formation of biotinylated DNA to the activity of *as* TET2.

To further assess the ability of the engineered pair to modulate the tandem hydroxymethylation–biotinylation process in a genome-wide manner, the entire genomic DNA isolated from HEK293T cells was treated with the *as* TET2 variant with or without **5**. The modified DNA was subjected to β-GT-mediated azidoglucosylation followed by "click" chemistry with **9**. The ligated product was directly analyzed by dot-blot assay using antibiotin antibody (Figure 5c). The chemiluminescent signal from the noninhibited sample confirmed catalytic activity of *as* TET2. Significantly reduced signal was observed when the TET assay was performed in the presence of NOG **5**. These results provide a blueprint for the potential application of *as* TET enzymes in probing genome-wide activity of a specific TET member when coupled to next-generation sequencing.

Allele-Specific Perturbation of TET Activity in Cultured Human Cells. The three human TET proteins, each with distinct structural features, have compensatory as well as nonoverlapping functions in cells. We sought to examine the effectiveness of the NOG-based inhibitors in modulating demethylase activity of the analogue-sensitive TET

DOI: 10.1021/jacs.8b05283
J. Am. Chem. Soc. 2018, 140, 10263–10269

enzymes in intact human cells. Toward this end, we synthesized octyl ester derivatives **10** and **11** of NOG **2** and leucine NOG **5**, respectively. It has been shown that esterification of 2KG and 2HG (2-hydroxyglutarate) improves their cell permeability (Figure 6a, Scheme S5).[42−44]

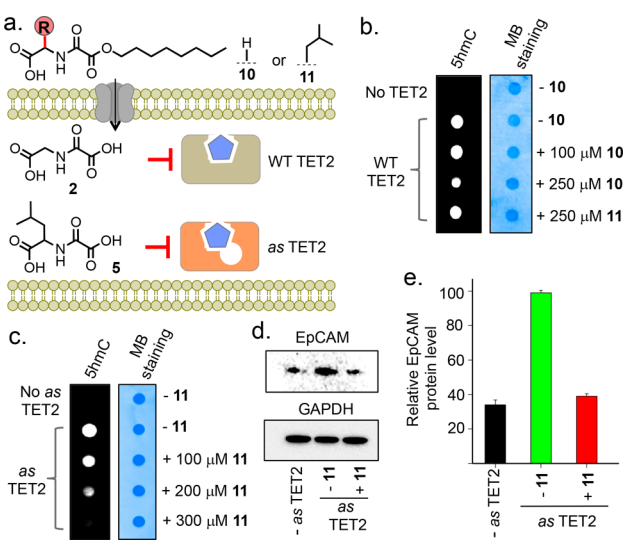


Figure 6. Cellular activity of *as* TET2 and NOG analogues. (a) Structures of cell-permeable NOG **10** and its "bumped" counterpart **11**; in HEK293T cells, they are hydrolyzed to **2** and **5**, respectively. (b) Dot-blot assay showing activity of wild-type TET2 on chromosomal DNA in cells. Its activity was inhibited by NOG **2** (hydrolyzed from **10**) but not by "bumped" NOG **5** (hydrolyzed from **11**). Methylene blue (MB) showing DNA staining as loading control. (c) Dot-blot assay showing dose-dependent inhibition of *as* TET2 by **5** (hydrolyzed from **11**) as measured by 5hmC formation on chromosomal DNA in cells. MB showing DNA staining as loading control. (d) Western blot analysis showing increased expression of EpCAM, but not GAPDH, by *as* TET2 enzyme. Selective inhibition of *as* TET2 by **5** (hydrolyzed from **11**) resulted in reduction of EpCAM expression. (e) Quantitative representation of Western blot data in (d) showing EpCAM expression decreased by 3-fold in the presence of NOG analogue **11** in cells.

Upon induced expression of the wild-type (or hole-modified) human TET2 catalytic domain in HEK293T cells, as confirmed by Western blot analysis (Figure S25), the genomic DNA was isolated and subjected to dot-blot assay. A robust level of 5hmC was observed only in cells expressing the enzymes and not in nontransfected cells, clearly demonstrating that both the enzymes are catalytically active in cells (Figure 6b,c). We observed a reduction in 5hmC level, albeit to a smaller extent, when cells expressing wild-type TET2 were treated with 100 and 250 μM of **10** (Figure 6b). This is consistent with the weaker inhibitory activity of NOG **2** (IC_{50} $\approx$ 150 μM) toward wild-type TET2.[26] However, no change in 5hmC content was noticed when these cells were cultured in the presence of 250 μM of cell-permeable "bumped" NOG analogue **11** (Figure 6b), further confirming our screening results that the "bumped" NOG **5** does not inhibit the wild-type enzyme.

Importantly, **11** strongly inhibited the *as* TET2 variant in cells in a dose-dependent manner, as evident from the reduction in 5hmC formation (Figures 6c, S26). A 100 μM concentration of the ester prodrug **11** was enough to reduce the 5hmC level to a significant degree (~75%) in these cells, consistent with the stronger inhibitory activity of **5** (IC_{50} $\approx$ 18 μM) toward *as* TET2. The lack of hydroxylase activity of *as* TET2 in these cells must arise from allele-specific inhibition by the NOG analogue **11**, as full recovery of TET2 activity ensued upon withdrawal of the compound from cell culture (Figure S26). These results demonstrate that the demethylase activity of TET2 variants on chromosomal DNA can be specifically and conditionally manipulated using a cell-permeable and reversible "bumped" NOG analogue in intact cells.

We also examined off-target activity of the NOG analogue **11** on 2KG-dependent endogenous histone demethylases. We extracted nuclear histones from HEK293T cells treated with 100 μM **11** and analyzed for four key trimethylated lysine residues in histone H3 ($H3K4Me_3$, $H3K9Me_3$, $H3K27Me_3$, and $H3K36Me_3$) using corresponding antibodies. Upon comparison with untreated cells, we did not observe any significant changes in histone methylation levels at these sites (Figure S27). This result further confirms the *in cellulo* selectivity of the "bumped" NOG analogue, making it suitable to study only the analogue-sensitive variant in human cells.

Gene regulatory function of TET enzymes is directly related to their catalytic ability to generate 5hmC in a loci-specific manner. Overexpression of TET2 is commonly associated with gene activation through the formation of 5hmC at promoters and enhancers. We sought to examine whether the *as* TET2 and the "bumped" inhibitor pair could modulate expression of certain genes in cells. We focused on the epithelial cellular adhesion molecule (EpCAM) and transducin beta like 1 X-linked (TBL1X) proteins, as it has been shown that shRNA-mediated knockdown of TET2 leads to the silencing of both *EPCAM* and *TBL1X* genes primarily via the loss of 5hmC at the promoter.[45,46] Consistently, overexpression of TET2 leads to reactivation of EpCAM and TBL1X proteins. Nuclear extracts from HEK293T cells expressing the *as* TET2 variant and treated with or without 100 μM **11** were collected and analyzed for the expression of both the proteins using appropriate antibodies. Overexpression of *as* TET2 led to an increase in EpCAM and TBL1X protein levels compared to the control cells lacking *as* TET2 (Figures 6d,e, S28). These results confirm that *as* TET2 is functionally competent in cells and required for expression of certain genes much like its wild-type counterpart. We observed, however, more than a 3-fold reduction in EpCAM and TBL1X proteins when *as* TET2-expressing cells grew in the presence of the "bumped" inhibitor **11** (Figures 6d,e, S28). Expression of housekeeping proteins such as GAPDH remained unaffected in all the samples. Collectively, our results show that bulky NOG inhibitor can modulate the gene regulatory functions of a specific TET member in human cells. Our study further demonstrates that selective inhibition of the catalytic activity of TET2 is sufficient to silence expression of certain genes.

■ CONCLUSION

The diversity-based mechanism of protein evolution, in which many protein products originate from relatively few genes, is central to the formation of closely related protein families. This has significantly hindered the development of isoform/member-specific chemical probes. In this piece of work, we employed structure-guided protein−ligand interface engineering to overcome the barrier imposed by active site degeneracy. TET mutants with a "hole" at the active site while still maintaining wild-type catalytic activity are specifically inhibited by designed NOG analogues carrying a complementary

DOI: 10.1021/jacs.8b05283
J. Am. Chem. Soc. 2018, 140, 10263−10269

"bump". These compounds do not interfere with the native TETs and a range of other 2KG-dependent enzymes and hence can serve as chemical probes for functional analysis of TET enzymes. Such specificity likely stems from a steric clash between the hydrophobic "bump" of the inhibitors and the conserved bulky gatekeeper residue in TET enzymes, as we have identified in the current study. Engineering at these gatekeeper residues in TET proteins extended the approach to all three members of the subfamily. Following successful probe development, we demonstrated their ability to conditionally manipulate 5hmC formation on genomic DNA. Furthermore, by combining the *as* technology with β-GT-mediated azido-glycosylation and bioorthogonal ligation, we developed a novel method for genome-wide mapping of TET member-specific activity. Finally, cell-permeable "bumped" NOG prodrug is shown to modulate the catalytic and transcriptional activity of the *as* TET2 variant in cultured human cells in a reversible manner, thus providing a means for conditional perturbation of DNA demethylases not attainable by either shRNA-mediated knockdown or gene knockout. We anticipate future experiments involving knock-in of *as* TET members into the endogenous *loci* in somatic as well as mouse embryonic stem cells employing CRISPR-Cas9 genome-editing technology for temporal manipulation of specific TET members with cell-permeable NOG analogues.[47,48] We foresee that our engineering approach for developing an analogue-sensitive demethylation apparatus will be amenable to the other members of the 2KG-dependent hydroxylase superfamily, particularly ones for which context-dependent functions are largely unknown.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b05283.

Synthetic procedure and characterization of all the relevant compounds, methods for protein expression and purification, and biochemical assay, supplementary figures and tables, and NMR spectra (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*kai27@pitt.edu

ORCID

Kabirul Islam: 0000-0002-8680-6130

Author Contributions

§V. Snyder, S. Kavoosi, and S. Arora contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the University of Pittsburgh and the NIH (R01GM123234) for financial support; Profs. Y. Xu, R. Trievel, C. Schofield, D. Fujimori, C. He, H. Leonhardt, and R. Kohli for the expression constructs used in the current study; Dr. D. Chakraborty and members of our laboratory for critical reading and editing of the manuscript. Support for MALDI-TOF MS instrumentation was provided by a grant from the National Science Foundation (CHE-1625002). This work is dedicated to Prof. Goverdhan Mehta.

■ REFERENCES

(1) Jeltsch, A. *Trends Biochem. Sci.* **2013**, *38*, 172.
(2) Loenarz, C.; Schofield, C. J. *Nat. Chem. Biol.* **2008**, *4*, 152.
(3) 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. *Science* **2009**, *324*, 930.
(4) Lu, X.; Zhao, B. S.; He, C. *Chem. Rev.* **2015**, *115*, 2225.
(5) Ito, S.; Shen, L.; Dai, Q.; Wu, S. C.; Collins, L. B.; Swenberg, J. A.; He, C.; Zhang, Y. *Science* **2011**, *333*, 1300.
(6) 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. *Science* **2011**, *333*, 1303.
(7) Schiesser, S.; Hackner, B.; Pfaffeneder, T.; Muller, M.; Hagemeier, C.; Truss, M.; Carell, T. *Angew. Chem., Int. Ed.* **2012**, *51*, 6516.
(8) Wu, S. C.; Zhang, Y. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 607.
(9) Oswald, J.; Engemann, S.; Lane, N.; Mayer, W.; Olek, A.; Fundele, R.; Dean, W.; Reik, W.; Walter, J. *Curr. Biol.* **2000**, *10*, 475.
(10) Ko, M.; Huang, Y.; Jankowska, A. M.; Pape, U. J.; Tahiliani, M.; Bandukwala, H. S.; An, J.; Lamperti, E. D.; Koh, K. P.; Ganetzky, R.; Liu, X. S.; Aravind, L.; Agarwal, S.; Maciejewski, J. P.; Rao, A. *Nature* **2010**, *468*, 839.
(11) Huang, Y.; Chavez, L.; Chang, X.; Wang, X.; Pastor, W. A.; Kang, J.; Zepeda-Martinez, J. A.; Pape, U. J.; Jacobsen, S. E.; Peters, B.; Rao, A. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 1361.
(12) Booth, M. J.; Raiber, E. A.; Balasubramanian, S. *Chem. Rev.* **2015**, *115*, 2240.
(13) Schreiber, S. L.; et al. *Cell* **2015**, *161*, 1252.
(14) Knight, Z. A.; Shokat, K. M. *Cell* **2007**, *128*, 425.
(15) Nishio, K.; Belle, R.; Katoh, T.; Kawamura, A.; Sengoku, T.; Hanada, K.; Ohsawa, N.; Shirouzu, M.; Yokoyama, S.; Suga, H. *ChemBioChem* **2018**, *19*, 979.
(16) Lopez, M. S.; Choy, J. W.; Peters, U.; Sos, M. L.; Morgan, D. O.; Shokat, K. M. *J. Am. Chem. Soc.* **2013**, *135*, 18153.
(17) Bishop, A. C.; Ubersax, J. A.; Petsch, D. T.; Matheos, D. P.; Gray, N. S.; Blethrow, J.; Shimizu, E.; Tsien, J. Z.; Schultz, P. G.; Rose, M. D.; Wood, J. L.; Morgan, D. O.; Shokat, K. M. *Nature* **2000**, *407*, 395.
(18) Carter-O'Connell, I.; Jin, H.; Morgan, R. K.; David, L. L.; Cohen, M. S. *J. Am. Chem. Soc.* **2014**, *136*, 5201.
(19) Gibson, B. A.; Zhang, Y.; Jiang, H.; Hussey, K. M.; Shrimp, J. H.; Lin, H.; Schwede, F.; Yu, Y.; Kraus, W. L. *Science* **2016**, *353*, 45.
(20) Belshaw, P. J.; Schoepfer, J. G.; Liu, K.-Q.; Morrison, K. L.; Schreiber, S. L. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2129.
(21) Lin, Q.; Jiang, F.; Schultz, P. G.; Gray, N. S. *J. Am. Chem. Soc.* **2001**, *123*, 11608.
(22) Baud, M. G.; Lin-Shiao, E.; Cardote, T.; Tallant, C.; Pschibul, A.; Chan, K. H.; Zengerle, M.; Garcia, J. R.; Kwan, T. T.; Ferguson, F. M.; Ciulli, A. *Science* **2014**, *346*, 638.
(23) Breski, M.; Dey, D.; Obringer, S.; Sudhamalla, B.; Islam, K. *J. Am. Chem. Soc.* **2016**, *138*, 13505.
(24) McDonough, M. A.; McNeill, L. A.; Tilliet, M.; Papamicael, C. A.; Chen, Q. Y.; Banerji, B.; Hewitson, K. S.; Schofield, C. J. *J. Am. Chem. Soc.* **2005**, *127*, 7680.
(25) Hu, L.; Li, Z.; Cheng, J.; Rao, Q.; Gong, W.; Liu, M.; Shi, Y. G.; Zhu, J.; Wang, P.; Xu, Y. *Cell* **2013**, *155*, 1545.
(26) Sudhamalla, B.; Dey, D.; Breski, M.; Islam, K. *Anal. Biochem.* **2017**, *534*, 28.
(27) Bishop, A. C.; Buzko, O.; Shokat, K. M. *Trends Cell Biol.* **2001**, *11*, 167.
(28) Islam, K. *ACS Chem. Biol.* **2015**, *10*, 343.
(29) Hu, L.; Lu, J.; Cheng, J.; Rao, Q.; Li, Z.; Hou, H.; Lou, Z.; Zhang, L.; Li, W.; Gong, W.; Liu, M.; Sun, C.; Yin, X.; Li, J.; Tan, X.; Wang, P.; Wang, Y.; Fang, D.; Cui, Q.; Yang, P.; He, C.; Jiang, H.; Luo, C.; Xu, Y. *Nature* **2015**, *527*, 118.
(30) Fu, Y.; Jia, G.; Pang, X.; Wang, R. N.; Wang, X.; Li, C. J.; Smemo, S.; Dai, Q.; Bailey, K. A.; Nobrega, M. A.; Han, K. L.; Cui, Q.; He, C. *Nat. Commun.* **2013**, *4*, 1798.

DOI: 10.1021/jacs.8b05283
J. Am. Chem. Soc. 2018, 140, 10263−10269

(31) Chen, W.; Zhang, L.; Zheng, G.; Fu, Y.; Ji, Q.; Liu, F.; Chen, H.; He, C. *FEBS Lett.* **2014**, *588*, 892.
(32) Williams, S. T.; Walport, L. J.; Hopkinson, R. J.; Madden, S. K.; Chowdhury, R.; Schofield, C. J.; Kawamura, A. *Epigenetics* **2014**, *9*, 1596.
(33) Krishnan, S.; Trievel, R. C. *Structure* **2013**, *21*, 98.
(34) Pack, L. R.; Yamamoto, K. R.; Fujimori, D. G. *J. Biol. Chem.* **2016**, *291*, 6060.
(35) Gerken, T.; Girard, C. A.; Tung, Y. C.; Webby, C. J.; Saudek, V.; Hewitson, K. S.; Yeo, G. S.; McDonough, M. A.; Cunliffe, S.; McNeill, L. A.; Galvanovskis, J.; Rorsman, P.; Robins, P.; Prieur, X.; Coll, A. P.; Ma, M.; Jovanovic, Z.; Farooqi, I. S.; Sedgwick, B.; Barroso, I.; Lindahl, T.; Ponting, C. P.; Ashcroft, F. M.; O'Rahilly, S.; Schofield, C. J. *Science* **2007**, *318*, 1469.
(36) Liu, M. Y.; Torabifard, H.; Crawford, D. J.; DeNizio, J. E.; Cao, X. J.; Garcia, B. A.; Cisneros, G. A.; Kohli, R. M. *Nat. Chem. Biol.* **2017**, *13*, 181.
(37) Lee, M.; Li, J.; Liang, Y.; Ma, G.; Zhang, J.; He, L.; Liu, Y.; Li, Q.; Li, M.; Sun, D.; Zhou, Y.; Huang, Y. *J. Am. Chem. Soc.* **2017**, *139*, 4659.
(38) Coulter, J. B.; O'Driscoll, C. M.; Bressler, J. P. *J. Biol. Chem.* **2013**, *288*, 28792.
(39) Song, C. X.; Clark, T. A.; Lu, X. Y.; Kislyuk, A.; Dai, Q.; Turner, S. W.; He, C.; Korlach, J. *Nat. Methods* **2012**, *9*, 75.
(40) Szwagierczak, A.; Bultmann, S.; Schmidt, C. S.; Spada, F.; Leonhardt, H. *Nucleic Acids Res.* **2010**, *38*, e181.
(41) Demko, Z. P.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2110.
(42) MacKenzie, E. D.; Selak, M. A.; Tennant, D. A.; Payne, L. J.; Crosby, S.; Frederiksen, C. M.; Watson, D. G.; Gottlieb, E. *Mol. Cell. Biol.* **2007**, *27*, 3282.
(43) Xu, W.; Yang, H.; Liu, Y.; Yang, Y.; Wang, P.; Kim, S. H.; Ito, S.; Yang, C.; Wang, P.; Xiao, M. T.; Liu, L. X.; Jiang, W. Q.; Liu, J.; Zhang, J. Y.; Wang, B.; Frye, S.; Zhang, Y.; Xu, Y. H.; Lei, Q. Y.; Guan, K. L.; Zhao, S. M.; Xiong, Y. *Cancer Cell* **2011**, *19*, 17.
(44) Zengeya, T. T.; Kulkarni, R. A.; Meier, J. L. *Org. Lett.* **2015**, *17*, 2326.
(45) Chen, J. Y.; Luo, C. W.; Lai, Y. S.; Wu, C. C.; Hung, W. C. *Oncogenesis* **2017**, *6*, e369.
(46) Wang, Y.; Xiao, M.; Chen, X.; Chen, L.; Xu, Y.; Lv, L.; Wang, P.; Yang, H.; Ma, S.; Lin, H.; Jiao, B.; Ren, R.; Ye, D.; Guan, K. L.; Xiong, Y. *Mol. Cell* **2015**, *57*, 662.
(47) Bartkowiak, B.; Yan, C.; Greenleaf, A. L. *Biochim. Biophys. Acta, Gene Regul. Mech.* **2015**, *1849*, 1179.
(48) Moyer, T. C.; Holland, A. J. *Methods Cell Biol.* **2015**, *129*, 19.

DOI: 10.1021/jacs.8b05283
J. Am. Chem. Soc. 2018, 140, 10263−10269