

Focused Screening Identifies Different Sensitivities of Human TET Oxygenases to the Oncometabolite 2-Hydroxyglutarate

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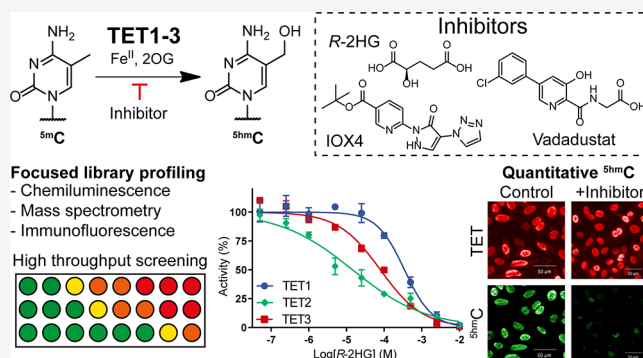
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ABSTRACT: Ten-eleven translocation enzymes (TETs) are Fe(II)/2-oxoglutarate (2OG) oxygenases that catalyze the sequential oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine in eukaryotic DNA. Despite their roles in epigenetic regulation, there is a lack of reported TET inhibitors. The extent to which 2OG oxygenase inhibitors, including clinically used inhibitors and oncometabolites, modulate DNA modifications via TETs has been unclear. Here, we report studies on human TET1–3 inhibition by a set of 2OG oxygenase-focused inhibitors, employing both enzyme-based and cellular assays. Most inhibitors manifested similar potencies for TET1–3 and caused increases in cellular ⁵hmC levels. (*R*)-2-Hydroxyglutarate, an oncometabolite elevated in isocitrate dehydrogenase mutant cancer cells, showed different degrees of inhibition, with TET1 being less potently inhibited than TET2 and TET3, potentially reflecting the proposed role of *TET2* mutations in tumorigenesis. The results highlight the tractability of TETs as drug targets and provide starting points for selective inhibitor design.



INTRODUCTION

Eukaryotic transcription is regulated by an array of epigenetic proteins that add, recognize, or remove covalent modifications on chromatin. DNA methyl transferases (DNMTs) introduce methyl groups on cytosine to produce 5-methylcytosine (⁵mC), a relatively stable epigenetic modification that is associated with repression or enhancement of transcription, depending on its genomic location.¹ The ten-eleven translocation enzymes (TETs) catalyze the sequential oxidation of ⁵mC to give 5-hydroxymethylcytosine (⁵hmC), 5-formylcytosine (⁵fC), and 5-carboxylcytosine (⁵caC) (collectively 5-oxidizedcytosines, ⁵oxC)^{2–4} (Figure 1A). The presence of ⁵mC at CpG islands in promoter regions is typically associated with transcriptional repression,⁵ whereas the roles of ⁵hmC appear to be context-dependent.^{6,7} ⁵oxC can be intermediates in demethylation of ⁵mC, either via passive dilution as a result of cell division,^{8,9} via active thymidine DNA glycosylase–base excision repair (TDG-BER)-mediated mechanism¹⁰ or putatively via direct deformylation of ⁵fC¹¹ (Figure 1A). ⁵oxC can have distinct and/or overlapping functions^{12,13} and are recognized by “reader” modules that bind to one or a combination of ⁵oxC marks to elicit biological responses.¹⁴ There is also evidence that ⁵fC and ⁵hmC may contribute to nucleosome stabilization and modulation of DNA stability.^{15–18}

TET-mediated oxidation of ⁵mC is catalyzed by Fe(II) containing oxygenase domains that use O₂ and 2-oxoglutarate (2OG) as cosubstrates (Figure 1A,B).⁴ Three human TET isozymes have been identified: TET1–3 (Figure 1C). Of the ~60–70 human 2OG oxygenases, several subfamilies are involved in transcriptional regulation, including the JmjC histone lysine demethylases (JmjC-KDMs) and the hypoxia-inducible factor (HIF) prolyl hydroxylase domains (PHDs), the latter of which act as O₂ sensors in responses to hypoxic stress.²⁰ All characterized 2OG oxygenases contain a distorted double-stranded β-helix (DSBH) core fold that supports Fe(II) binding by a conserved HXD/E...H triad (Figure 1B).²¹ In the TETs, two DSBH β-strands (β12 and β13) are split by a characteristic low complexity insert (LCI) (Figure 1C).¹⁹ A cysteine-rich domain, situated adjacent to the oxygenase domain, is proposed to stabilize the DSBH fold and is necessary for TET1–3 catalysis.¹⁹ TET1 and TET3 contain a CXXC-domain that preferentially binds to nonmethylated

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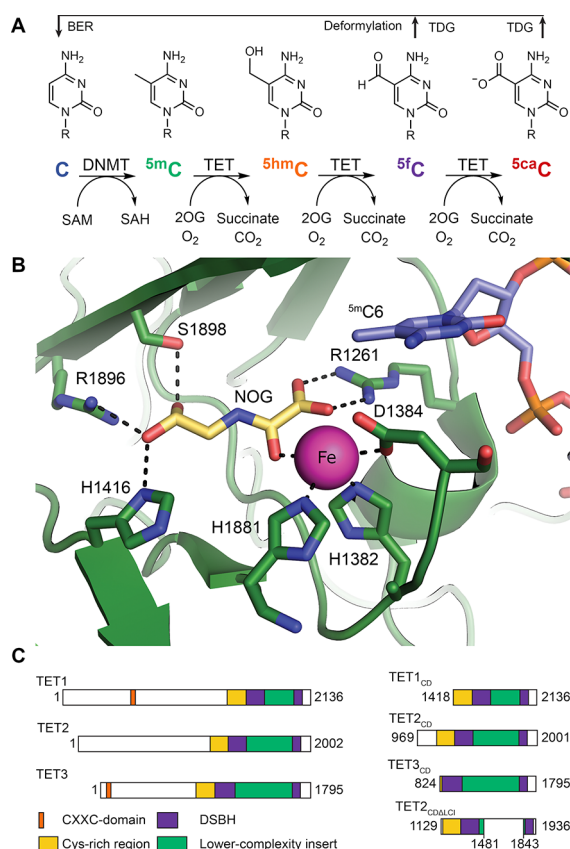


Figure 1. 2OG oxygenase catalytic domains of TETs catalyze sequential oxidation of ^{5m}C to ^{5hm}C, ^{5f}C, and then ^{5ca}C. (A) Schematic representation of cytosine methylation, oxidation, and demethylation cycle. Cytosine is methylated by DNMTs to give ^{5m}C, which undergoes sequential oxidation to ^{5hm}C, ^{5f}C, and ^{5ca}C, as catalyzed by TETs. ^{5f}C and ^{5ca}C can be restored to C by the TDG-BER mechanism. (B) View of the active site of TET2 (green) complexed with ^{5m}C-containing DNA (blue). ^{5m}C is positioned near Fe(II) (pink) and 2OG binding sites (cofactor mimic NOG, yellow) (the figure was derived from PDB: 4NM6¹⁹). Dark blue represents nitrogen; red as oxygen; and orange as phosphorus. (C) TET domain structures (left) and TET constructs used in this study (right).

DNA.²² TET2 lacks this domain (Figure 1C) but binds to several DNA-binding factors, including IDAX (CXXC4) which is reported to enable DNA binding in a similar manner as the CXXC-domain in TET1 and TET3.²³ The TETs catalyze the oxidation of ^{5m/ox}C substrates in DNA and RNA and thymidine in DNA²⁴ with varying efficiencies depending on the context of the substrate base, including whether it is in single- or double-stranded DNA and the presence of other modifications in RNA.^{25–27}

TET mutations are linked to diseases, inter alia hematopoietic malignancies such as chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), and chronic myelomonocytic leukemia (CMML).^{28–30} TET2 is among the most frequently mutated genes in myeloid neoplasms and inactivating TET2 mutants are linked to DNA hypermethylation, tumor progression, and poor patient outcome. In AML and myelodysplastic syndrome (MDS), TET2 mutations are mostly mutually exclusive with those in isocitrate dehydrogenase (*IDH1/2*),³¹ which catalyzes decarboxylation of isocitrate to give 2OG. Mutant *IDHs* catalyze the production of the oncometabolite (2*R*)-hydroxyglutarate (*R*-

2HG), which can accumulate to high levels (up to 30 mM).^{32,33} Elevated 2HG levels are proposed to inhibit 2OG oxygenases, including the TETs, in cells.³⁴ The mutually exclusive nature of *IDH* and *TET2* mutations in AML is proposed to be due to functional redundancy³¹ in tumorigenesis and/or the synthetic lethality of TET1/3 inhibition by *R*-2HG in *TET2* mutant cells, which are reliant on TET1/3 for survival.³⁵ TET inhibition is thus a potential strategy for treatment of AML-bearing *TET2* mutants. Genetic studies have also identified TET2 inhibition as a potential way to improve cancer immunotherapy.³⁶

Potent and selective inhibitors will aid in functional studies and evaluation of the TETs as therapeutic targets. Some TET inhibitors are reported,^{37–40} including metal-chelating 2OG cofactor mimics, such as TCA cycle-related metabolites, including (*S*)- and (*R*)-2HG,^{34,41–43} IOX1,³⁷ 2,4-PDCA,⁴¹ and TETi76;³⁵ however, knowledge of their selectivity, potency, and cell activities across TET1–3 is limited. The development of TET inhibitors has likely been limited to date by a paucity of assays with sufficient sensitivity and throughput.³⁸ Here, we report on the development of robust TET assays, both with isolated enzymes and in cells, which were used to screen a set of 2OG oxygenase-focused inhibitors and related compounds. We observed that several known 2OG oxygenase inhibitors also inhibit TET1–3, including some clinically used PHD inhibitors. Unexpectedly, *R*-2HG and *S*-2HG had different inhibition potencies across the TET1–3, as manifested in studies with isolated enzymes and in cells.

RESULTS

Quantitative Assay for the Detection of ^{5hm}C by TET-Mediated ^{5m}C Oxidation. To develop a quantitative assay for kinetic studies and inhibitor screening of isolated TET1–3, the catalytic domain (CD) of recombinant human TET2_{CD} (Q969-I2002) was produced in insect cells (Sf9), and TET3_{CD} (E824-I1795) was produced in mammalian cells. TET1_{CD} (E1418-V2136), which was produced in insect (Sf9) cells, was from a commercial source (Figures 1C and S1). To monitor TET activity, an AlphaScreen assay was developed,³⁷ wherein the biotinylated ^{5hm}C product from a quenched TET reaction was quantitated by a ^{5hm}C-antibody, using streptavidin conjugated donor and Protein-A acceptor bead pairs (Figure S2A).³⁷ A standard curve with 32-base single-stranded 5'-biotinylated ^{5m}C-1, ^{5hm}C-2, and ^{5m}C 1/^{5hm}C 2-DNA mixture demonstrated the assay is sensitive and selective for ^{5hm}C relative to ^{5m}C (>10 S/N at ≥0.1 nM) with a linear range up to 1.5 nM ($R^2 = 0.98$) (Figure S2B). Time course assays with recombinant TETs showed an increase in the ^{5hm}C signal in an enzyme concentration-dependent manner; assay saturation was observed at higher enzyme concentrations, possibly due to further oxidation of ^{5hm}C (Figure S3A–C). While TET1_{CD} (0.005 μM min⁻¹ μM⁻¹) and TET3_{CD} (0.016 μM min⁻¹ μM⁻¹) had comparable activities, the specific activity of TET2_{CD} was >10-fold higher (0.21 μM min⁻¹ μM⁻¹); thus, 10 nM protein was used for TET1/3_{CD} assays, while TET2_{CD} was sufficiently active at 1 nM (Figure S3A–C). The K_M^{app} (2OG) values of TETs measured using AlphaScreen were 2.4 ± 0.3 μM (TET1_{CD}) and 11.1 ± 3.5 μM (TET2_{CD}) under nonsaturating substrate conditions (10 nM ^{5m}C-DNA 1, Figure S4), which are similar to reported K_M (2OG) of 15.7 ± 2.8 μM for TET2_{CDΔLCI} using a mass spectrometry (MS) method.⁴²

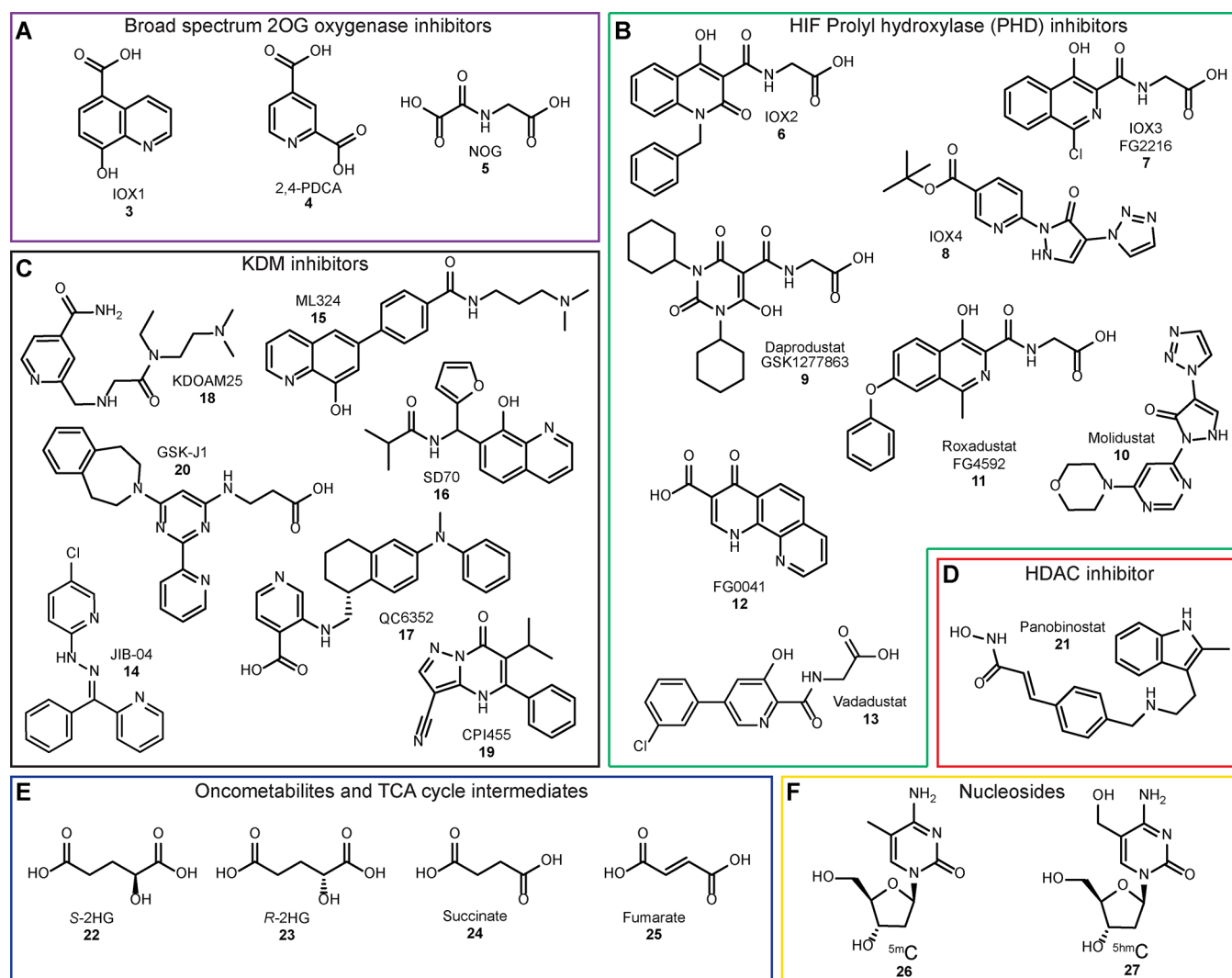


Figure 2. Small molecule inhibitors tested against TET1–3. The focused panel contains broad-spectrum 2OG oxygenase inhibitors (A, purple), HIF PHD inhibitors (B, green), KDM targeted inhibitors (C, black), HDAC inhibitor (D, red), TCA cycle intermediates and related compounds (E, blue), and relevant nucleosides (F, yellow).

Inhibitor Screening against TET Enzymes Using the AlphaScreen Assay. To investigate the selectivity of TET inhibition, a set of 2OG oxygenase–focused inhibitors, including broad-spectrum inhibitors,^{44,45} PHD-selective inhibitors,^{46,47} JmjC-KDM-selective inhibitors,⁴⁸ (2R) and (2S)-hydroxyglutarate³³ (*R/S*-2HG), TCA cycle metabolites and other epigenetic protein inhibitors,⁴⁹ was tested using the AlphaScreen method³⁷ (Figure 2). IOX1 3, a broad-spectrum 2OG oxygenase inhibitor and relatively potent inhibitor of TET1/2,³⁷ was used as a positive control. As most reported 2OG oxygenase inhibitors are 2OG competitors and contain an Fe(II) chelating group,²⁰ IC₅₀ screens were carried out at approximate K_M^{app} of 2OG (10 μM) (Figure S4) in the linear ranges using TET1 (10 nM), TET2 (1 nM), or TET3 (10 nM). Most tested compounds inhibited TET1–3 to varying degrees (Table 1 and Figure 3A–F).

The broad-spectrum inhibitors (purple box, Figure 2), such as IOX1 3, 2,4-pyridine-dicarboxylic acid (2,4-PDCA, 4), and *N*-oxalylglycine (NOG, 5), were approximately equipotent inhibitors of TET1–3, with IC₅₀ values in the low micromolar range ($\text{pIC}_{50} = \sim 5\text{--}6$). While IOX1 3 inhibition was similar to that reported,³⁷ NOG 5 inhibited TET1_{CD}, TET2_{CD}, and

TET3_{CD} with IC₅₀ values of 13, 9, and 7 μM , respectively, an order of magnitude more potent than reported for TET2_{CDALCI} using an MS assay (IC₅₀ = 149 \pm 8 μM ; enzyme concentration: 5 μM).⁴² The PHDs are validated therapeutic targets, with several inhibitors for them being approved or in development for treatment of anemia associated with chronic kidney disease^{47,49} (Figure 2). Weak or no inhibition (IC₅₀ \geq 100 μM , $\text{pIC}_{50} \leq 4$) of TETs was observed for Daprodustat (GSK1277863, 9), Roxadustat (FG4592, 11), and their analogues IOX2 6 and IOX3 (FG2216, 7).⁴⁷ Molidustat (BAY85-3934, 10) also showed little inhibition, but its analogue IOX4 8 was a moderately potent inhibitor of TET1_{CD} and TET2_{CD} with IC₅₀ values of 17 and 19 μM , respectively. FG0041 12 and Vadadustat (AKB-6548, 13) were the most potent TET inhibitors of the PHD inhibitors tested, with low single-digit micromolar IC₅₀ values (Table 1).

JIB-04 14 and ML324 15 (Figure 2), which were initially reported as selective JmjC-KDM inhibitors⁵⁰ but were subsequently shown to have wider inhibitory profiles,^{51,52} and showed here relatively potent TET1–3 inhibition (Table 1). JmjC-KDM inhibitors with higher KDM selectivity, i.e., GSK-J1 20,^{53,54} QC6352 17,^{55,56} KDOAM25 18,⁵⁷ and

Table 1. Results of Inhibitors Screened against the Human TETs^a

compound		AlphaScreen pIC ₅₀				SPE-MS pIC ₅₀	Cellular pEC ₅₀	Cellular pCC ₅₀
		TET1 _{CD}	TET2 _{CD}	TET3 _{CD}	TET2 _{CDΔLCI}	TET2 _{CDΔLCI}	TET1 _{CD}	TET1 _{CD}
IOX1	3	6.08 ± 0.64	5.31 ± 0.24	5.57 ± 0.19	5.59 ± 0.26	5.76 ± 0.05	4.99 ± 0.32 (12)	<3
2,4-PDCA	4	5.77 ± 0.79	5.29 ± 0.07	6.11 ± 0.10	5.01 ± 0.10	5.82 ± 0.09	<3 ^b (4)	<3 ^b
NOG	5	4.87 ± 0.21	5.05 ± 0.19	5.16 ± 0.01	5.12 ± 0.58	6.30 ± 0.10	3.96 ± 0.42 ^b (10)	<3 ^b
IOX2	6	61% ± 16	32% ± 11	N.I.	33% ± 3	4.78 ± 0.02	≤3 (2)	<3
IOX3	7	43% ± 18	42% ± 7	47% ± 2	46% ± 21	5.29 ± 0.05	<3 (2)	<3
IOX4	8	4.76 ± 0.39	4.73 ± 0.03	64% ± 9	4.80 ± 0.18	4.52 ± 0.19	4.10 ± 0.32 (13)	3.01 ± 0.42
Daprodustat	9	63% ± 25	31% ± 6	36% ± 10	27% ± 12	4.38 ± 0.02	<3 (1)	N.D.
Molidustat	10	N.I.	N.I.	60% ± 3	N.I.	N.I.	~4 (1)	<3.7
Roxadustat	11	25% ± 6	44% ± 13	30% ± 16	N.I.	N.D.	<3 (3)	N.D.
FG0041	12	5.42 ± 0.26	5.26 ± 0.53	N.D.	5.33 ± 0.01	5.25 ± 0.02	4.62 ± 0.29 (2)	<3.7
Vadadustat	13	5.20 ± 0.24	5.30 ± 0.01	5.26 ± 0.03	5.21 ± 0.02	5.69 ± 0.06	<3 (4)	N.D.
JIB-04	14	5.13 ± 0.20	5.52 ± 0.01	5.01 ± 0.12	5.45 ± 0.01	5.2% ± 2	6.36 ± 0.42 (4)	5.01 ± 0.66
ML324	15	5.89 ± 0.21	5.81 ± 0.10	5.90 ± 0.04	6.09 ± 0.04	4.74 ± 0.18	4.28 ± 0.42 (5)	4.14 ± 0.17
SD70	16	35% ± 29	47% ± 4	55% ± 7	54% ± 1	58% ± 14	N.D.	N.D.
QC6352	17	28% ± 1	30% ± 8	29% ± 2	62% ± 2	34% ± 6	N.D.	N.D.
KDOAM25	18	N.I.	N.I.	N.I.	30% ± 6	N.I.	<3 (2)	<3.7
CPI455	19	N.I.	N.I.	N.I.	N.I.	N.I.	N.D.	N.D.
GSK-J1	20	26% ± 9	22% ± 2	N.D.	N.I.	N.D.	4.80 ± 0.56 ^b (4)	3.94 ± 0.26 ^b
Panobinostat	21	5.38 ± 0.30	5.42 ± 0.31	5.33 ± 0.03	5.68 ± 0.01	4.62 ± 0.11	<3 ^c (4)	~8
S-2HG	22	2.98 ± 0.05	4.89 ± 0.06	3.98 ± 0.17	5.21 ± 0.07	5.87 ± 0.52	N.I. ^b (3 mM) (3)	<2.7 ^b
R-2HG	23	3.17 ± 0.24	4.81 ± 0.03	4.02 ± 0.11	5.14 ± 0.35	5.93 ± 0.47	2.83 ± 0.09 ^b (3)	<2.7 ^b
succinate	24	3.72 ± 0.27	3.86 ± 0.18	3.61 ± 0.05	3.98 ± 0.31	4.83 ± 0.35	2.46 ± 0.10 ^b (3)	2.27 ± 0.16 ^b
fumarate	25	3.93 ± 0.14	3.69 ± 0.08	3.71 ± 0.03	3.80 ± 0.35	5.03 ± 0.23	3.50 ± 0.15 ^b (3)	3.57 ± 0.15 ^b
^{5m} C	26	N.D.	N.D.	N.D.	N.D.	N.I.	N.D.	N.D.
^{5hm} C	27	N.D.	N.D.	N.D.	N.D.	N.I.	N.D.	N.D.
dimethyl-2OG	33	N.D.	N.D.	N.D.	N.D.	N.D.	2.17 ^b	<2 ^b

^apIC₅₀, pEC₅₀, and pCC₅₀ values of small molecule inhibitors tested against human TET1–3 using AlphaScreen, SPE-MS assays or cellular IF assays. Values of enzyme assays are indicated as pIC₅₀, with incomplete dose–response curves noted as % inhibition at 100 μM. Inhibition of cellular TET1 activity was measured as pEC₅₀ values with independent biological replicates in parentheses (*n*). pCC₅₀ values are based on the nuclear count measured by DAPI staining. N.I. indicates no inhibition at 100 μM (<20%), N.D.: not determined. Values are mean ± StDev, *n* = 2–5. See Figure 2 for compound structures and Figure S10 for prodrug structures. AlphaScreen IC₅₀ not determined for nucleosides ^{5m}C and ^{5hm}C due to antibody assay interference. ^bEster prodrug used. ^cApparent increase of ^{5hm}C levels.

CPI455 **19**,⁵⁸ were either very weak inhibitors (IC₅₀ > 100 μM) or did not inhibit TET1–3. SD70 **16**, a reported inhibitor of ligand and genotoxic stress-induce translocations in prostate cancer and which was later identified to inhibit KDM4C,⁵⁹ was a weak TET inhibitor (35–55% at 100 μM SD70 **16**). The broad-spectrum histone deacetylase (HDAC) inhibitor Panobinostat **21** (red box, Figure 2), an approved drug for multiple myeloma by the U.S. Food and Drug Administration and European Medicines Agency, inhibited TET1–3 (IC₅₀ = 3.8–4.7 μM) albeit with a steep hill slope for TET1_{CD} (>4); note that hydroxamic acids chelate Fe(II) and are reported to inhibit some 2OG oxygenases including JmjC-KDMs.^{44,60,61}

The oncometabolite R-2HG (D-2HG, **23**) is reported to inhibit the TETs.³⁴ Despite the weak observed inhibition using recombinant murine and human TETs (murine Tet1/2 IC₅₀ = 4–5 mM^{34,43} and human TET2_{CDΔLCI} IC₅₀ = 5.3 mM,⁴² Table S2), it was proposed that elevated levels of R-2HG **23** in IDH mutant cells may be sufficient to impact on global ^{5hm}C levels via TET inhibition.³⁴ In the AlphaScreen assay, S- and R-2HG **22**, **23** were found to be very weak inhibitors of TET1_{CD} (IC₅₀ = ~0.8 mM, for both enantiomers, respectively). Unexpectedly, both S- and R-2HG **22**, **23** were moderate and weak inhibitors of TET2_{CD} (IC₅₀ = 13–15 μM, respectively) and TET3_{CD} (IC₅₀ = ~100 μM for both 2HG enantiomers), respectively (Table 1). Thus, while the 2HG potencies differed for different TET subfamily members, S- and R-2HG **22**, **23**

manifested equal potency versus the individual TET enzymes. The TCA cycle intermediates succinate **24** and fumarate **25** were weak inhibitors of TET1–3 (IC₅₀ = 118–245 μM), in accord with results for murine Tet1/2.⁴³

LCI in the Catalytic Domain of TET2_{CD} Has Little Impact on the Inhibition Profile. The above-mentioned results reveal TET1–3 shares relatively similar inhibition profiles, with the exceptions of S- and R-2HG **22**, **23** (Figure 3I–K). The differential 2HG potencies are particularly evident when comparing inhibition of TET1_{CD} and TET2_{CD} by both S- and R-2HG **22**, **23**; both enantiomers are >43-fold more potent for TET2_{CD} than TET1_{CD}. The apparent discrepancy in our IC₅₀ values and those reported for TET2_{CDΔLCI},⁴² a TET2 construct without the LCI (Figure 1C), prompted us to investigate the effect of the LCI on TET catalysis and inhibition. Recombinant TET2_{CDΔLCI} (D1129–G1936 with S1481–N1843 replaced by a 3 × GGGGS linker) was produced in *E. coli* and purified as reported,¹⁹ yielding relatively high levels of purified protein (~1.6 mg L⁻¹, > 85% purity by SDS-PAGE) (Figures 1C and S1). TET2_{CDΔLCI} showed similar activity to TET2_{CD} (specific activity = 0.14 μM min⁻¹ μM⁻¹, K_M^{app} (2OG) = 4.9 ± 0.3 μM) (Figures S3D and S4) and its inhibition profile correlated with that of TET2_{CD} (Pearson rank = 0.96, Spearman rank = 0.89) (Table 1, Figures 2 and 3G,H,L). S-2HG **22** and R-2HG **23** inhibit TET2_{CDΔLCI} with single-digit micromolar potency (S-2HG **22** IC₅₀ = 6.0

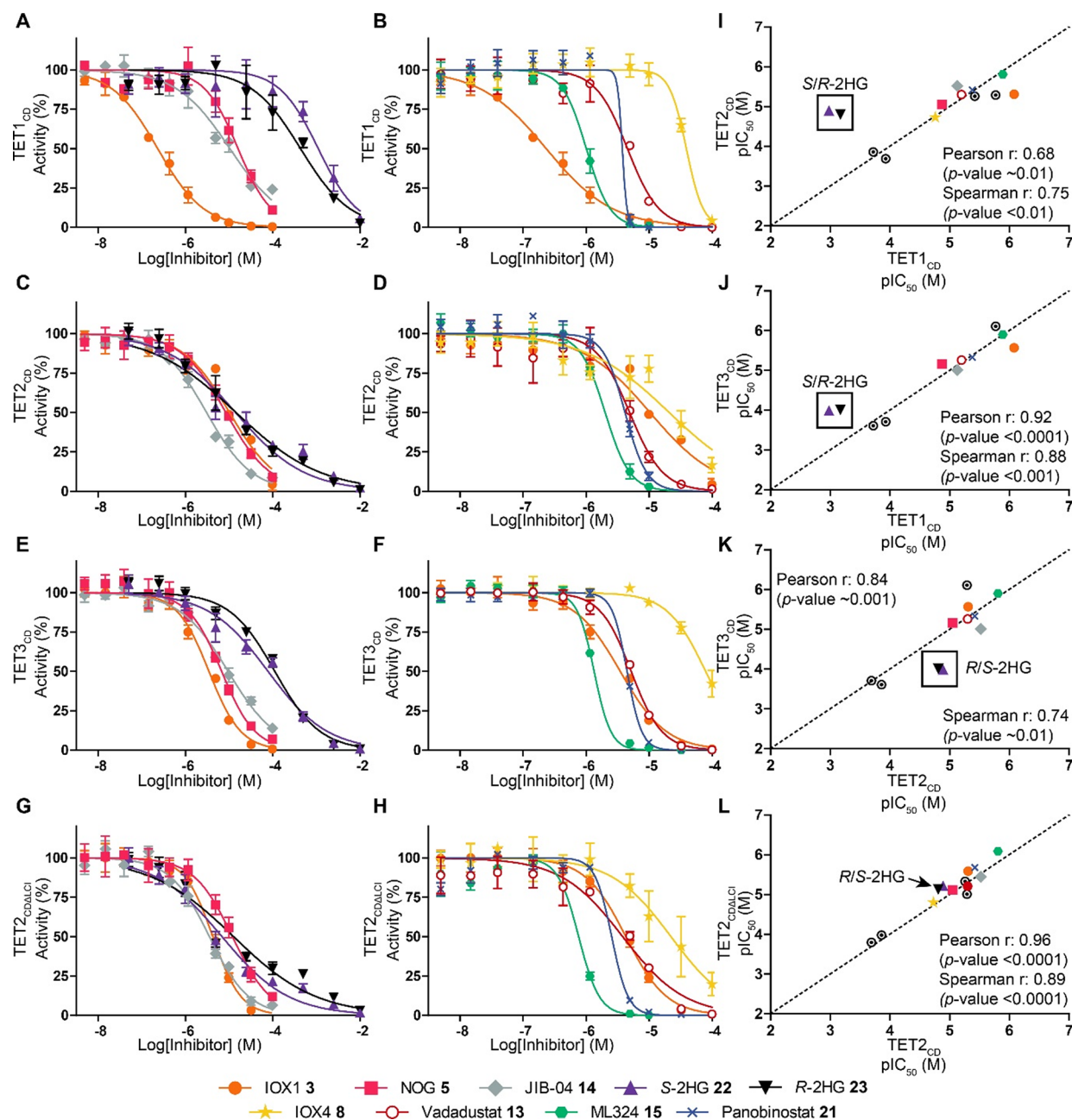


Figure 3. Representative AlphaScreen IC₅₀ curves and correlation curves for inhibitors tested against TET1–3_{CD} and TET2_{CDΔLCI}. Plots for TET1_{CD} (A,B), TET2_{CD} (C,D), TET3_{CD} (E,F), and TET2_{CDΔLCI} (G,H) of selected inhibitors are shown. IOX1 3 (orange), NOG 5 (pink), JIB-04 14 (gray), S-2HG 22 (purple), R-2HG 23 (black), ML324 15 (green), Vadadustat 13 (red), IOX4 8 (yellow) and Panobinostat 21 (blue). I–L AlphaScreen pIC₅₀ correlation plots for inhibitors for TET1–3_{CD}, TET2_{CDΔLCI}. Pearson correlation and Spearman coefficients were calculated for comparisons. Standard conditions: ^{5m}C (1, 10 nM), ascorbate (100 μM), Fe(II) (10 μM), with TET1_{CD} (10 nM, 30 min incubation), TET2_{CD} (1 nM, 10 min incubation), TET3_{CD} (10 nM, 10 min incubation), or TET2_{CDΔLCI} (1 nM, 10 min incubation). *n* = 2–4, error given as ± StDev. IC₅₀ values are displayed in Table 1; compound structures are given in Figure 2. Circled dots—other compounds from Table 1.

μM and R-2HG 23 IC₅₀ = 7.2 μM). Further kinetic studies with both TET2 enzymes using AlphaScreen assays revealed IOX1 3 to be a 2OG competitive inhibitor (Table S1 and Figure S5), consistent with studies on other 2OG oxygenases.⁴⁵ S-2HG 22 and R-2HG 23 showed similarly potent 2OG competitive inhibition with TET2_{CD} (S-2HG 22 *K_i* = 19 μM, R-2HG 23 *K_i* = 23 μM) and TET2_{CDΔLCI} (S-2HG 22 *K_i* = 6 μM, R-2HG 23 *K_i* = 12 μM) (Table S1 and Figure S5B,C). These observations suggest that the presence of the LCI has little impact on the inhibition profile, at least under our assay conditions. TET2_{CDΔLCI} is thus a good model enzyme for

kinetic studies for TET2 due to its ease of production and was used in subsequent MS assays, which have higher enzyme requirements than the AlphaScreen assay. For TET1_{CD}, the *K_i* values of S-2HG 22 and R-2HG 23 were 269 and 246 μM, respectively (Table S1), which were notably less potent (>10-fold) than against TET2_{CD/CDΔLCI}.

Mass Spectrometry-Based Assays for the Detection of TET Activity on Double-Stranded DNA. While the AlphaScreen method provides a high-throughput, sensitive assay with low reagent requirements, the indirect immunobead-based detection can lead to assay interference and false

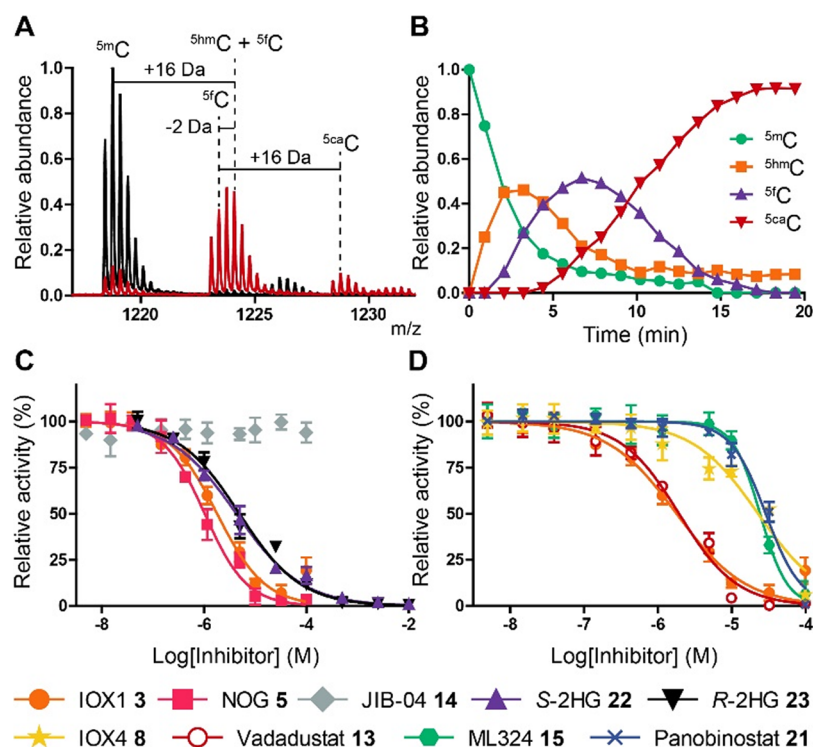


Figure 4. Measurement and quantitation of TET2_{CDΔLCl} catalyzed oxidation of ^{5m}C to ^{5hm}C, ^{5f}C, and ^{5ca}C using the SPE-MS assay. (A) Representative SPE-MS spectra for TET2_{CDΔLCl} (0.8 μM) catalysis in the presence of ^{5m}C 28 (1.0 μM) using the [M-3H]³⁻ charge state. Overlay of spectra from 0 min (black) and 5.57 min (red) time points showing sequential oxidation of ^{5m}C to ^{5hm}C (+16 Da relative to ^{5m}C), ^{5f}C (+14 Da relative to ^{5m}C), and ^{5ca}C (+30 Da relative to ^{5m}C). (B) Corresponding time course displaying the relative abundance of ^{5m}C 28 (green), ^{5hm}C (orange), ^{5f}C (purple), and ^{5ca}C (red). (C,D) Representative IC₅₀ curves of IOX1 (3, orange), NOG (5, pink), JIB-04 (14, gray), S-2HG (22, purple), R-2HG (23, black), IOX4 (8, yellow), Vadadustat (13, red), ML324 (15, green), and Panobinostat (21, blue) tested with TET2_{CDΔLCl} (0.4 μM) and ^{5m}C-DNA 28 (2.0 μM). Assays were quenched after 10 min (~20–30% ^{5hm}C product formation at a linear range (R^2 : 0.99, Figure S6C)) to minimize formation of subsequent oxidative products ^{5f}C and ^{5ca}C. Standard conditions: ^{5m}C-DNA (28, 2.0 μM) and TET2_{CDΔLCl} (0.4 μM), ascorbate (200 μM), Fe(II) (50 μM), 2OG (10 μM). Data are plotted as mean ($n = 2-4$) and error given as \pm StDev.

positives. ssDNA, a possibly less efficient TET substrate than dsDNA,²⁷ was used in AlphaScreen assays because it yielded significantly higher signals than dsDNA, possibly due to increased accessibility of the ^{5hm}C-antibody to ^{5hm}C-DNA. Thus, an MS assay was developed to directly measure substrate depletion/product formation using dsDNA. Reported MS methods for TET assays, including for kinetic studies, are typically based on LC-MS/MS which employ oligonucleotide hydrolysis to nucleosides for analysis.³⁸ However, the LC-MS methods require higher amounts of substrate/enzymes and can require downstream processing steps. To improve sample throughput and assay resolution, we developed a method consisting of solid-phase extraction coupled to MS assay (SPE-MS), as has been applied to other 2OG oxygenases.⁶² The SPE-MS method enables fast (<12 s) sample processing, near real-time monitoring of TET-mediated ^{5m}C oxidation and is based on a reported method.⁶³ The SPE-MS assay was optimized with the palindromic 12-base pair ^{5m}C-DNA (ACCAC^{5m}CGGTGGT, 28) with TET2_{CDΔLCl}, which enabled good sensitivity and robust detection of activity. A time course for TET2_{CDΔLCl} in the presence of ^{5m}C-DNA 28 resulted in the resolution of substrate and products, including all three oxidized states of cytosine (Figure 4A,B). To measure ^{5hm}C and ^{5f}C, which have a 2 Da mass difference and overlapping isotopic patterns, peak area extraction was carried out to estimate their quantities relative to ^{5m}C and ^{5ca}C (Figure 4A). ^{5m}C was observed to be sequentially oxidized in a time-dependent manner to give ^{5hm}C, then ^{5f}C, and finally ^{5ca}C

(Figure 4B). After 20 min ^{5ca}C-DNA was the major product observed (~90%), followed by ^{5hm}C-DNA (~10%), under our assay conditions. No evidence for oxidation of a potential thymidine substrate to 5-hydroxymethyluracil (^{5hm}U), a reported TET product,²⁴ was detected. Kinetic analysis of TET2_{CDΔLCl} with saturating ^{5m}C-DNA 28 (i.e., above the K_M 28 of 1 μM as determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS,⁴² Figure S6A) yielded K_M^{app} (2OG) of 3.0 ± 0.6 μM (Figure S6B), similar to that observed with the AlphaScreen assay (Figure S4).

Inhibitor Screening against TET2_{CDΔLCl} Proteins Using SPE-MS. The TET2_{CDΔLCl} inhibition profile using SPE-MS (Table 1, Figures 4C,D and 6C) was in general agreement with the AlphaScreen results (Table 1 and Figure S6D). IOX1 3 gave a similar level of inhibition ($IC_{50} = 1.7$ μM) relative to AlphaScreen. Interestingly, in the SPE-MS assay NOG 5, a near 2OG isostere, showed increased potency ($\Delta pIC_{50} = +1.18$), while JIB-04 14 ($\Delta pIC_{50}^{est} = -1.43$), ML324 15 ($\Delta pIC_{50} = -1.35$) and Panobinostat 21 ($\Delta pIC_{50} = -1.06$) gave reduced potency. The difference in potencies observed between the two assays (Pearson correlation coefficient r : 0.29; Spearman correlation coefficient r : 0.15, Figure S6D) could, in part, reflect the increased Fe(II) concentration in the SPE-MS compared to the AlphaScreen assay (50 and 10 μM, respectively).⁵⁰ SPE-MS enabled the analysis of ^{5m}C 26 and ^{5hm}C 27 nucleosides as potential inhibitors, but no inhibition was observed at 100 μM, indicating that the oligomeric DNA context is important in TET binding. Importantly, the SPE-MS

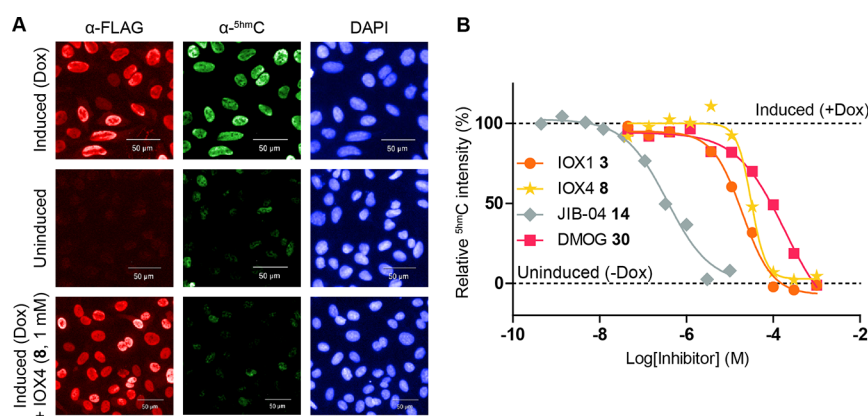


Figure 5. Small molecule inhibitors of TETs can reduce global ⁵hmC levels in cells. (A) Selected images of IF staining of Dox-inducible U2OS cells stably transfected with FLAG-tagged TET1_{CD}. An increase in FLAG (red) and ⁵hmC (green) staining, corresponding to overexpression of catalytically active TET1_{CD}, is observed only after Dox (1 mg mL⁻¹)-mediated induction. DAPI nuclear staining is in blue. Reduction in the ⁵hmC level is observed while FLAG staining is maintained when cells are treated with TET inhibitors (e.g., IOX4 8). This trend corresponds to observations with cells overexpressing a catalytically inactive TET1_{CD} mutant (Figures S7–S9). (B) Representative EC₅₀ curves for IOX1 3, IOX4 8, JIB-04 14, and DMOG 30 for Dox-induced U2OS cells overexpressing TET1_{CD}. All tested compounds reduce ⁵hmC levels in a dose-dependent manner. The ⁵hmC levels of Dox-induced and -uninduced control cells (1% DMSO) are indicated. Data are plotted as mean and error given as \pm s.e.m ($n > 3000$ cells). See Figures S9 and S11 for dosing data on TET1_{CD} MUT.

results confirmed both S- and R-2HG 22, 23 are relatively potent TET2_{CDALCI} inhibitors.

Cellular Inhibition of TETs by Small Molecules. To evaluate the effect of TET inhibition in cells, we developed an immunofluorescence (IF) assay to measure changes in genomic ⁵hmC levels as a function of TET1 activity. Stable U2OS cells with doxycycline (Dox)-inducible FLAG-tag wild-type (WT) TET1_{CD} and a catalytically inactive variant (MUT, H1672A/D1674A) TET1_{CD} were generated (Figure S7). FLAG staining was observed only in Dox-induced cells and colocalized with DAPI in the nucleus, indicating TET1_{CD} was expressed. Dox-dependent TET1_{CD} expression was shown by Western blots (Figure S7A–C). Next, U2OS cells were induced with Dox for 24 h, fixed, and stained with either ⁵hmC- and FLAG-antibodies, fluorescence-conjugated secondary antibodies or DAPI. The cells were visualized and quantitated using high-content imaging (Figure 5A). Substantial staining for ⁵hmC was detected in Dox-induced WT TET1_{CD} overexpressing cells, while minimal background ⁵hmC levels were detected in uninduced and MUT TET1_{CD} overexpressing cells (Figures 5A, S8A,B and S9A), implying that the WT TET1_{CD} efficiently catalyzes oxidation of ⁵mC to ⁵hmC in cells.

Next, we assessed inhibitors (Figure 2) or prodrug equivalents (Figure S10) in U2OS cells. Compounds were dosed for 24 h with simultaneous induction of TET1_{CD} expression, and IF analysis of FLAG and ⁵hmC levels was performed (Figure 5 and Table 1). Uninduced TET1_{CD}WT and induced TET1_{CD}MUT were used as negative controls (Figures S9 and S11). Of the broad-spectrum inhibitors, IOX1 3 was relatively potent against TET1_{CD} (EC₅₀ = 10 μ M), while dimethyloxalylglycine (DMOG, 30, prodrug of 5) (Figure 5B) was a weaker inhibitor (EC₅₀ = 110 μ M) and dimethyl 2,4-PDCA (31, prodrug of 4) showed no inhibition at 1 mM. In agreement with the isolated enzyme assays, most of the PHD-selective inhibitors (IOX2 6, IOX3 7, Daprodustat 9) were not TET1_{CD} inhibitors in cells, except for Molidustat 10 where weak inhibition (EC₅₀ = \sim 100 μ M) was observed. IOX4 8, an analogue of Molidustat 10 showed similar inhibitory activity in cells (EC₅₀ = 79 μ M). Interestingly, Vadadustat (AKB-6548,

13) inhibited TETs at single-digit micromolar potency in enzyme assays but exhibited no measurable inhibition in the cellular assay. FG0041 (12) showed both activity in both isolated enzyme and cellular assays (EC₅₀ = 24 μ M). Note there is no information on the relative efficiencies of cellular uptake/efflux/metabolism of these inhibitors in U2OS cells so the differences between TET inhibition on isolated enzymes and of TET1_{CD} in cells may, in part, reflect these factors.

Out of the KDM inhibitors tested, JIB-04 14 and ML324 15 were the only compounds that inhibited TET1_{CD} with both the isolated enzyme and cellular assays. JIB-04 14 was the most potent TET inhibitor in cells, with an EC₅₀ of 0.44 μ M, which is substantially more potent than observations with the AlphaScreen enzyme assay (IC₅₀ = 7.4 μ M). Although no changes in ⁵hmC levels were observed with TET1_{CD}MUT, JIB-04 14 induced a concentration-dependent decrease in cell numbers (CC₅₀ = \sim 10 μ M); thus, nonspecific mechanisms causing toxicity cannot be ruled out. Similar trends have been observed for JIB-04 14 treatment of JmjC KDM overexpressing HeLa cells, suggesting a likely promiscuous mechanism for JIB-04 14.⁵² GSK-J4 (32, prodrug of 20) showed apparent cell activity at EC₅₀ = 16 μ M, while exhibiting no inhibition against recombinant TET1_{CD}. Interestingly, increased levels of ⁵hmC were observed when cells were dosed with the HDAC inhibitor Panobinostat 21, despite being a moderate inhibitor of isolated TET1_{CD} (IC₅₀ = 4.2 μ M) (Figure S12). Increased staining of ⁵hmC was observed below 11 μ M (up to 1.5-fold relative to the DMSO control) with a concomitant increase in FLAG staining/TET1 expression, followed by a rapid reduction in ⁵hmC levels at $>$ 33 μ M, correlating with a dose-dependent reduction in cell counts (Figures S11 and S12). Prodrugs of TCA cycle intermediates, dimethyl esters of 2OG 33, succinic acid 34, and fumaric acid 35, were also tested. Dimethyl 2OG 33 and dimethyl succinate 34 were very weak potential TET1_{CD} inhibitors in cells (EC₅₀ = \sim 6.7 and \sim 3.5 mM, respectively). Dimethyl fumarate 35 inhibited with an EC₅₀ of 316 μ M (Table 1), but also induced cell death at the same concentration range (CC₅₀ = 270 μ M). While both S- and R-2HGs have similar potencies against isolated recombinant

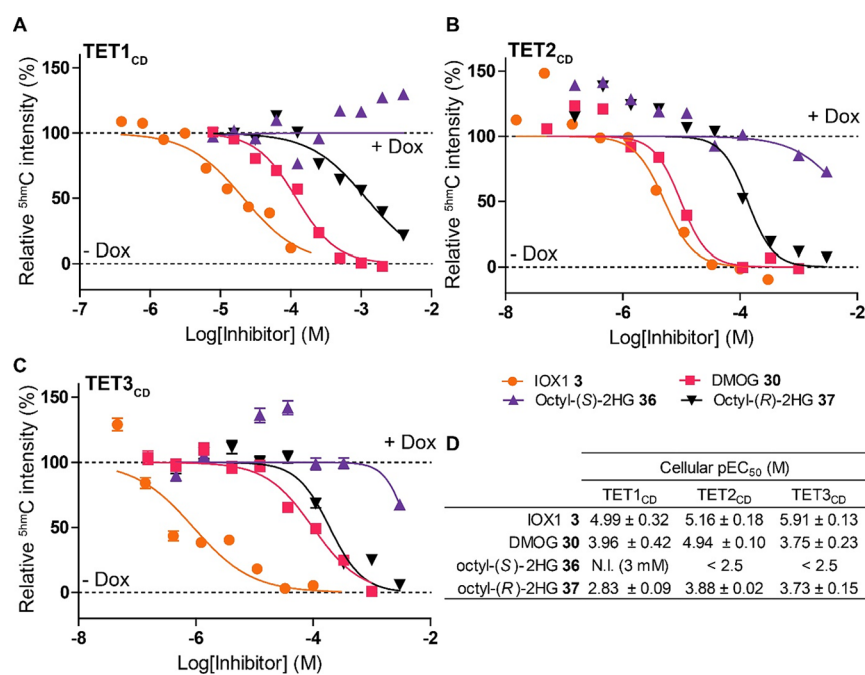


Figure 6. Evidence that the TET isozymes have different sensitivities to 2-hydroxyglurate enantiomers (2HGs) in cells. Representative dose-response curves for IF cell assays for U2OS cells stably expressing TET1_{CD} (A), TET2_{CD} (B), and TET3_{CD} (C) dosed with inhibitors for 24 h. Data are normalized to ⁵hmC levels of doxycyclin-induced TET-expressing cells (+Dox) and uninduced (-Dox) cells treated with 1% DMSO. Data are plotted as mean, and the error is given as \pm s.e.m ($n > 3000$ cells). (D) Tabulated cellular pEC₅₀ values of inhibitors in IF assays for TET1_{CD}, TET2_{CD}, and TET3_{CD}. Data are shown as mean with error given as \pm StDev. of independent biological replicates with the number of replicates in brackets. N.I.: no inhibition at 3 mM. See Figure S13 for associated FLAG staining, cell counts, and TET1_{CD} MUT data.

TET1, their prodrugs showed different activities in cells. Octyl-ester *R*-2HG 37 had very weak inhibitory activity on TET1 in cells ($\sim 50\%$ inhibition at 1.5 mM), and no inhibition was observed for octyl-ester of *S*-2HG 36 at 3 mM (Table 1 and Figure 6A).

To investigate the differential sensitivities of TET subfamily members to 2HG observed in isolated enzymes, we generated stable U2OS cell lines with Dox-inducible expression of FLAG-tag WT TET2_{CD} and TET3_{CD}. After dox-dependent TET gene expression was confirmed by Western blot and IF staining (Figures S7D,E and S8C,D), TET2_{CD} and TET3_{CD} cells were treated with IOX1 3, DMOG 30 or octyl-ester 2HGs 36, 37 and analyzed by IF. IOX1 3 was a potent inhibitor for both TET2_{CD} and TET3_{CD} ($EC_{50} = 6.9 \mu\text{M}$ and $1.2 \mu\text{M}$, respectively). DMOG 30 manifested EC_{50} values of 11 and $177 \mu\text{M}$, for TET2_{CD} and TET3_{CD}, respectively, a similar range of inhibition observed for TET1_{CD}. The octyl-ester of *R*-2HG 37 showed modest, but clear, inhibition of both TET2_{CD} ($EC_{50} = 131 \mu\text{M}$) and TET3_{CD} ($EC_{50} = 186 \mu\text{M}$) in cells (Figure 6B–D). Similar to the results for TET1, the octyl-ester *S*-2HG 36 poorly inhibited TET2_{CD} or TET3_{CD} in cells (up to 3 mM octyl-ester *S*-2HG 36 tested). Thus, the trends of recombinant TET inhibition reflect the cellular inhibition for the *R*-2HG 37, that is, *R*-2HG is a more potent inhibitor of TET2 and TET3, than TET1.

DISCUSSION AND CONCLUSIONS

We have developed robust, sensitive, and quantitative assay platforms for the discovery of inhibitors for TET1–3. Using complementary AlphaScreen and MS-based assays, we found the 2OG K_M values for TET1/2 to be in the micromolar range, in agreement with reported studies⁴² and similar to reported values for many, but not all, 2OG oxygenases^{45,64} (Table S2).

These assays were used to screen a focused set of 2OG oxygenase inhibitors against isolated catalytic domains of TET1–3, using 2OG at its K_M value and using low enzyme concentrations (nM). Consistent with reports, broad-spectrum 2OG oxygenase inhibitors, including IOX1 3, 2,4-PDCA 4, and NOG 5, were inhibitors of TET1–3. Our IC₅₀ values are generally lower than those reported,⁴² reflecting the increased sensitivity of our assays that allow lower enzyme concentrations to be used (Table 1).

Under physiological conditions, intracellular 2OG concentrations (0.6–2.7 mM^{65,66}) are estimated to be significantly above the calculated K_M for 2OG of the TETs (2–11 μM , Figure S4). Treatment of TET1 overexpressing cells with a prodrug of 2OG (dimethyl 2OG, 33) showed no enhancement in ⁵hmC levels (Table 1), an observation which implies that the TET activity is not limited by 2OG availability under our cellular assay conditions. Most of the inhibitors identified in the screens with isolated TET1–3 CDs (or corresponding ester-prodrugs) showed cellular TET activity, with a dose-dependent reduction in the TET-catalyzed oxidation of ⁵mC to ⁵hmC, albeit in general with higher EC_{50} than IC₅₀ values. For some compounds, cellular activity was not observed despite inhibition being observed with isolated TET(s), a discrepancy which might reflect low cell permeability, compound degradation, or rates of prodrug ester hydrolysis. Of particular interest, the PHD inhibitor Vadadustat 13,⁶⁷ developed for the treatment of anemia secondary to chronic kidney disease, was found to be among the most potent inhibitors of the TETs (IC₅₀ = $\sim 5 \mu\text{M}$) in enzyme assays, although activity was not observed in cell assays. However, it is possible that low levels of TET inhibition may contribute to the off-target effects associated with Vadadustat 13, although it should be noted that the cellular relevance of our work is unclear at this stage.

Interestingly, the KDM inhibitors JIB-04 **14** and GSK-J1/4 20/32 were the most potent cellular TET1 inhibitors identified, with submicromolar efficacy, but were less potent in the isolated TET assays. This difference may reflect effects resulting from dual KDM and TET inhibition; however, the lack of selectivity of JIB-04 **14** in cells may reflect inhibition of multiple 2OG oxygenases or effects on other relevant enzymes or variables such as iron or oxygen availability.⁶⁸ In contrast to its inhibitory effect against the TETs, the HDAC inhibitor Panobinostat **21** appeared to increase ^{5hm}C levels in cells in a concentration and TET1 activity-dependent manner. HDAC inhibition is shown to result in elevated levels of histone acetylation, an epigenetic mark associated with euchromatin. HDAC inhibition may improve the accessibility of DNA to TET1 as well as increase TET1 expression.

Most of the identified inhibitors were active versus all of TET1–3, likely in part, reflecting their 2OG competitive nature and conservation of the TET active sites. Both in isolated form and in cells, TET1–3 were inhibited by IOX1 **3** and NOG **5**, or its cell-penetrating derivative DMOG (Table 1 and Figure 6). DMOG is widely used in cellular studies and in animal models as inhibitors of 2OG oxygenases and as a hypoxia mimic. As suggested,⁷⁰ some of the effects of DMOG may reflect inhibition of 2OG oxygenases other than the PHDs, including TET inhibition. The TCA cycle metabolites succinate **24** and fumarate **25** have also been shown to (weakly) inhibit several 2OG oxygenases, and were similarly weak (sub-mM) inhibitors of the TETs in our assays, in agreement with IC₅₀ values reported for mouse Tet1/2.⁴³ Exogenous treatment of succinate **24** and fumarate **25** prodrugs led to dose-dependent reductions in cellular TET1 activity and global reduction in ^{5hm}C levels, albeit with concomitant cytotoxicity, so it is uncertain whether the effects are directly due to TET inhibition. Loss-of-function mutations of succinate dehydrogenase (SDH) and fumarate hydratase (FH) are found in multiple cancers^{71,72} and result in accumulation of high concentrations of succinate and fumarate, respectively, which are proposed to promote tumorigenesis; such elevated metabolite levels have been proposed to lead to reduced global ^{5hm}C levels through TET inhibition.^{43,73,74}

Importantly, our results provide unique and clear evidence for differential sensitivity to the oncometabolite R-2HG **23** for TET1–3; in isolated enzymes, TET2 is most sensitive ($K_i = 12\text{--}23\ \mu\text{M}$; enzyme concentration: 1 nM), while TET3 was weakly inhibited (IC₅₀ = ~100 μM ; enzyme concentration: 10 nM), and TET1 was poorly inhibited at sub-mM to mM concentrations (Figure 3 and Table 1). The observations imply stronger inhibition of TET2 by R- and S-2HG ($K_i = 6\text{--}23\ \mu\text{M}$) than previously reported using the same protein construct (TET2_{CD Δ LCl} R-2HG and S-2HG IC₅₀ = 5.3 mM and 12.4 mM, $K_i^{\text{calc}} = 0.9\ \text{mM}$ and 2.1 mM, respectively) or related murine isoforms ($K_i^{\text{calc}} = 0.2\text{--}1.3\ \text{mM}$, Table S2). These differences may, at least in part, be due to the sensitivity of our assays. TET2 inhibition of R-2HG ($K_i = 12\text{--}23\ \mu\text{M}$) is among the more potent values for inhibition of 2OG oxygenases by R-2HG, with KDM4A ($K_i = 13\ \mu\text{M}$ ⁷⁵) and KDM2A (IC₅₀ = 106 μM ⁷⁵ and $K_i^{\text{calc}} = 74\ \mu\text{M}$) also manifesting potent inhibition. In contrast, other tested 2OG oxygenases such as PHD2 ($K_i = 625\ \mu\text{M}$ ⁷⁶), ABH2 (IC₅₀ = 424 μM ⁷⁵ and $K_i^{\text{calc}} = 212\ \mu\text{M}$), or KDM5B (IC₅₀ = 203 μM ⁷⁷ and $K_i = 10\ \text{mM}$ ³⁴) are weakly inhibited (Table S2). It should be noted that the precise assay conditions can influence potency of 2OG oxygenase inhibition.⁷⁸ Nonetheless, in cellular assays, a prodrug form

of R-2HG **37** showed similar trends in relative inhibition of the TET1–3 to that observed with isolated TET1–3 enzymes, with TET2 being the most sensitive isozyme (EC₅₀ of TET2 (132 μM) < TET3 (186 μM) < TET1 (~1.5 mM)). Little or no inhibition in cells was observed for the prodrug form of S-2HG **36** at 3 mM. The inactivity of S-2HG **36** may, in part, be due to poor prodrug hydrolysis and/or cellular uptake, or, less likely, differences in the intracellular dehydrogenases acting on the 2HG clearance⁷⁹ (e.g., D2HGDH⁸⁰ vs L2HGDH⁸¹). Inhibition of TETs by R-2HG is shown to result in aberrant ^{5m}C/^{5ox}C patterns in AML, and TET2 may be particularly affected due to its abundance and sensitivity. Elevated cellular R-2HG (and S-HG) levels are also found in 2-hydroxyglutaric aciduria, a rare neurometabolic disorder resulting from gain-of-function IDH2 mutations or mutations in the D2HGDH (or L2HGDH) gene.⁸² Thus, while elevated R-2HG levels are likely to also affect other 2OG oxygenases, the available evidence suggests that TET2 may be particularly sensitive to cellular inhibition by R-2HG (Table S3).

Overall, the results demonstrate that TET1–3 are tractable targets for small molecule inhibition and provide starting points for structure–activity relationship (SAR) studies to obtain selective TET inhibitors. The results highlight the importance of inhibitor cross-screening against TET1–3 and suggest that it should be possible to obtain inhibitors selective for individual TET isozymes. This is demonstrated by the results showing less potent R-2HG inhibition of TET1 compared to TET3 and, in particular, TET2. Future work can also focus on determining the relevance of the different sensitivities of the TETs to R-2HG in cancer.

EXPERIMENTAL SECTION

General Reagents. Chemicals were from commercial sources unless stated otherwise. 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES, Apollo Scientific Limited, Code: B18181), sodium chloride (Fisher Bioreagents, Cat#: BP358-10), bovine serum albumin (BSA, PerkinElmer, Stabilizer, 7.5% (DTPA-purified BSA), Cat: CR84-100), Tween 20 (Promega, ref#: H5151), disodium 2-oxoglutarate (Sigma, Cat#: K3752-100G), (+)-sodium L-ascorbate (Sigma, Cat#: 11140-50G), (NH₄)₂Fe(II)-(SO₄)₂ (Sigma-Aldrich, cat#: 215406-100G), ethanediaminetetraacetate disodium salt dihydrate (EDTA, Sigma, cat#: E5134-500G), ^{5hm}C-specific antibody (Active Motif Cat#: 39769), AlphaScreen beads (PerkinElmer, IgG Detection Kit (Protein A), Cat#: 6760617M, lot: 1571768), Complete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics Ltd.), ProxiPlate -384 (White, Shallow 384 well, Pinch bar design, part#: 6008280), and DMSO (Fisher Chemical, code: D/4120/PB08). Water, LiChroSolv for LC-MS (VWR cat#: (1.15333.2500)), acetonitrile (LiChroSolv for LC, VWR, cat#: 1.00030.2500). N-oxalylglycine disodium was prepared according to the reported procedure.^{85,84}

Inhibitors. IOX1, IOX2, ML324, KDMOAM25, succinate, fumarate, 2OG, dimethylpyridine-2,4-dicarboxylate, dimethyl 2-oxoglutarate, dimethyl succinate, dimethyl fumarate, and DMOG were purchased from Sigma-Aldrich, while IOX3, IOX4, Roxadustat, Vadadustat, Panobinostat, disodium 2S/2R-hydroxyglutarate, and FG0041 were obtained from Cayman Chemical Company. 2,4-PDCA (Alfa Aesar), Daprodustat (MedChemExpress), QC6352 (MedChemExpress), Molidustat (Selleck Chem), JIB-04 (Tocris Bioscience), GSK-J1 (Tocris Bioscience), GSK-J4 (Tocris Bioscience), SD70 (Xcess Biosciences), CPI455 (Axon Med Chem), 2'-deoxy-5-methylcytidine (TCI Chemicals), 2'-deoxy-5-(hydroxymethyl)cytidine (TCI Chemicals), and (R/S)-Octyl- α -hydroxyglutarate (Cambridge Bioscience) were purchased from other commercial sources. All purchased inhibitors were >95% pure as determined by the manufacturer. Key compound purity was

verified using analytical HPLC using a UV detector (210 or 254 nm) or CAD detector (Figures S18–S28). NOG was synthesized as described in the materials and methods and has a purity >95% as determined by HPLC. All buffer components were from Sigma-Aldrich unless otherwise stated.

AlphaScreen Inhibitor Assays. Single-stranded 32-bp DNA ([Biotin]-5'-TCG GAT GTT GTG GGT CAG CGC ATG ATA GTG TA-3'), where C is ^{5m}C or ^{5hm}C , was from ATDBio (Oxford, UK). The AlphaScreen General IgG detection kit was from PerkinElmer. All enzyme reactions were performed in assay buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 0.1% BSA, 0.01% Tween 20). TET enzyme in assay buffer (5 μ L) was dispensed into ProxiPlate wells and preincubated with inhibitors for 10 min. The DNA-cofactor solution (20 nM DNA, 200 μ M sodium L-ascorbate (Asc), 20 μ M $(NH_4)_2Fe(II)(SO_4)_2$, 20 μ M disodium 2OG in buffer) was dispensed (5 μ L) to initiate the enzyme reaction and incubated at room temperature. Reactions were quenched with 30 mM EDTA (pH 4.2, 5 μ L). The AlphaScreen beads mixture (5 μ L) (preincubated (30 min) acceptor and donor AlphaScreen beads (62.5 \times dilution) with ^{5hm}C -specific antibody (Active Motif Cat#: 39769, 1:4000 dilution)] was added with incubation for 45 min, followed by analysis using a PerkinElmer EnVision (2104 Multilabel Reader). Data were processed using Microsoft Excel (2010, version 14.0) and GraphPad Prism 5 (v. 5.04). IC_{50} curves were calculated using GraphPad as log(inhibitor) vs normalized response–variable slope fit. AlphaScreen inhibition assays were conducted at different final protein concentrations and reaction times depending on the construct: TET1_{CD} (10 nM, 30 min), TET2_{CD} (1 nM, 10 min), TET2_{CD Δ LICI} (1 nM, 10 min), and TET3_{CD} (10 nM, 10 min).

SPE-MS Assays. TET2_{CD Δ LICI} (0.8 μ M) in buffer (50 mM HEPES, pH 7.0) (12.5 μ L) was dispensed into inhibitor plates and preincubated for 10 min. The DNA substrate (4 μ M 5'-ACC AC ^{5m}C GGT GGT-3' (ATDBio, Oxford, UK), 400 μ M sodium L-ascorbate, 20 μ M disodium 2-oxoglutarate, and 100 μ M $(NH_4)_2Fe(II)(SO_4)_2$ in buffer) (12.5 μ L) was then dispensed across the plate to initiate the reaction. The enzyme reaction was progressed for 10 min and the reaction was quenched by dispense of 2 mM NOG (25 μ L). Samples were analyzed using an Agilent RapidFire RF360 high-throughput sampling robot coupled to an Agilent 6530 accurate-mass quadrupole time-of-flight (Q-TOF) mass spectrometer (see below for details). Data were processed using Masshunter Qualitative Analysis Version B.0 7.00 and Agilent RapidFire Integrator software. IC_{50} values were calculated using GraphPad Prism 5 (v. 5.04) using the model log(inhibitor) vs normalized response–variable slope fit.

IF Assay for ^{5hm}C . Stable U2OS cell lines with tetracycline/Dox-inducible expression of TET catalytic domains (TET1_{CD} (1481–2136 aa), TET2_{CD} (1129–2002 aa), and TET3_{CD} (824–1795 aa) with N-terminal 3 \times FLAG) were generated using the Flp-In-T-Rex system (Invitrogen). For TET1_{CD}, the construct contained C-terminal GFP, and the catalytically inactive mutant TET1_{CD} (H1672Y and D1674A) was generated. Stable U2OS cells were maintained in DMEM media (Sigma) supplemented with TET system-approved fetal bovine serum (10%, FBS, Clontech), penicillin G (50 IU mL $^{-1}$, Invitrogen), streptomycin (50 μ g mL $^{-1}$, Invitrogen), L-glutamine (2 mM, Sigma), blasticidin S (5 μ g mL $^{-1}$, Invitrogen), and hygromycin B (150 μ g mL $^{-1}$, Roche Applied Science). Prior to transfection, cells were seeded into clear-bottom 96-well plates (CellCarrier Ultra-96, PerkinElmer, 5000 cells per well), in DMEM media supplemented with Tet system-approved FBS (10%), penicillin G (50 IU mL $^{-1}$), streptomycin (50 μ g mL $^{-1}$), and L-glutamine (2 mM), and allowed to adhere for 4 h at 37 $^{\circ}C$. Cells were then simultaneously dosed with compounds (1% DMSO final concentration) and Dox HCl (1 μ g mL $^{-1}$). Cells were then incubated (24 h) at 37 $^{\circ}C$, rinsed with phosphate-buffered saline (PBS; Gibco), then fixed in paraformaldehyde for 20 min (4%, Alfa Aesar) and permeabilized with TritonX-100 (0.5%, Sigma, 8 min) in PBS. Prior to blocking, the fixed cells were treated with HCl_(aq) (2 N) for 30 min, followed by the removal and neutralization of excess acid on cells with 100 mM Tris HCl (pH 8.0) (Sigma) for 15 min. Cells were then blocked with FBS (3%) in PBS for 30 min, and incubated with a primary antibody solution in

FBS (3%) in PBS for 16 h at 4 $^{\circ}C$ (anti- ^{5hm}C rabbit polyclonal antibody (Active Motif cat#: 39769, 1:500 dilution), anti-FLAG mouse monoclonal antibody (Sigma F1804, 1:1000 dilution)). The cells were washed with PBS, then incubated with a secondary antibody in FBS (3%) in PBS for 1 h (antirabbit Alexa-647 conjugate and antimouse Alexa-568 conjugate, Life Technologies, 1:500 dilution). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Cell imaging was performed using Operetta CLS High-Content Analysis (PerkinElmer) or a Cell Discoverer 7 high-throughput (Zeiss) systems. Images were analyzed using Harmony high-content imaging and analysis software (PerkinElmer). ^{5hm}C staining intensities (Alexa Fluor 647) of FLAG-TET expressing cells postcompound treatment were determined (mean fluorescence, s.e.m., and N). Nonlinear regression (four parameters) with constraints (Dox-induced and uninduced DMSO treated WT cells as top and bottom respectively) was used to calculate pIC₅₀ (–log (IC₅₀/M)) using Prism 7 (Ver 7.01, Graphpad Software, Inc.). Data are given as mean pIC₅₀ \pm s.e.m. of at least three independent biological replicates. pCC₅₀ values calculated based on the cell number (DAPI nuclear staining) within a set of analyzed fields.

Antibodies Used in the Study. For Western blots, the following primary antibodies were diluted in BSA (5%) in 1 \times PBS buffer: monoclonal ANTI-FLAG M2 antibody produced in mouse (1:1000, F1804, Merck); anti- β -actin antibody, mouse monoclonal (1:2000, A1978, Merck). HRP goat antirabbit IgG antibody (peroxidase) (1:3000, PI-1000, Vectorlabs), and peroxidase antimouse IgG (H+L) (affinity purified) (1:3000, PI-2000, Vectorlabs) were used as secondary antibodies.

For IF assays, the following primary antibodies were diluted in blocking buffer (FBS 3% in 1 \times PBS): the 5-hydroxymethylcytosine (^{5hm}C) antibody (pAb) (1:500, Active Motif cat#: 39769) and monoclonal ANTI-FLAG M2 antibody produced in mouse (1:500, F1804, Merck). Goat antimouse Alexa Fluor 568 (1:500, A-11031, Invitrogen) and Goat antirabbit Alexa Fluor 647 (1:500, A-21236, Invitrogen) were used as secondary antibodies.

Recombinant Protein Production. TET1_{CD}. Recombinant TET1_{CD} (L1418-V2136) with an N-terminal 3 \times Flag-tag produced in Sf9 cells was purchased from Epigentek (Cat#: E12002-1). The purity was confirmed by SDS-PAGE (Figure S1).

TET2_{CD}. Human TET2 (Q969-Y2001) was cloned by ligation-independent cloning into baculovirus transfer vector pFB-CT10HF-LIC (Addgene plasmid #39191). Bacmid DNA was generated by Tn7 recombination in the DH10Bac cell line and transfected into Sf9 cells with the JetPrime reagent (Polyplus). P2 virus was used to infect 6 L of Sf9 cells in Insect-XPRESS medium (Lonza) and TET2_{CD}-His₁₀-FLAG protein was produced over 72 h at 27 $^{\circ}C$ with shaking at 100 rpm. The cells were harvested and suspended in buffer (10 mM imidazole, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), 500 mM NaCl, 5% glycerol (v/v)] in 100 mM HEPES (pH 7.4)), and protease inhibitors set III (Calbiochem) was added. Cells were lysed using a Dounce homogenizer; the clarified lysates were incubated with Nickel-Sepharose 6 FF (GE Healthcare) for 1 h at 4 $^{\circ}C$ with rotation. After gravity column transfer, the resin was successively washed with buffer containing increasing imidazole concentrations (50, 70, and 100 mM, and the protein was eluted with buffer containing 250 mM imidazole. Eluted fractions containing purified protein of the desired mass (as judged by SDS-PAGE assay) were concentrated, injected onto a size exclusion Superdex 200 16/60 (GE healthcare) column, and eluted in gel filtration buffer (0.5 mM TCEP, 5% (v/v) glycerol, 500 mM NaCl in 20 mM HEPES (pH 7.4)). Fractions containing TET2_{CD}-His₁₀-FLAG protein of the correct molecular weight as determined using SDS-PAGE (Figure S1), concentrated, and used for assay development and inhibitor screening.

TET3_{CD}. The TET3 gene (E824–I1795) was cloned into pHTBV1.1-CT10H–SIII-LIC (C-terminal His₁₀-Twin-Strep-TEV) vector. The bacmid containing the TET3 insert (E824–I1795) was used to transfect Sf9 cells (Sf9 *Spodoptera frugiperda* cells for virus amplification, Expi293F cells for recombinant protein production) to produce baculovirus for infection. The virus was amplified by growing Sf9 cells (Insect EXPRESS medium from Lonza, 2 \times 10⁶ cells mL $^{-1}$)

incubated in shaker flasks. Cells were shaken at 90 rpm at 27 °C for 60 h. The cell suspensions were centrifuged (15 min, 800 × g, 4 °C); the supernatant containing the amplified TET3 virus was stored at 4 °C. Expi293F GnTI- cell cultures (1 L, 2 × 10⁶ cells mL⁻¹) in Freestyle 293 Expression medium (ThermoFisher Scientific) were infected with the baculovirus in the presence of sodium butyrate (5 mM) in a roller bottle (2 L). Cells were shaken in a humidity-controlled incubator for 72 h at 37 °C under a partial CO₂ (8%) atmosphere. The cell suspensions were harvested by centrifugation (15 min, 800 × g, 4 °C) and the cell pellet was resuspended in PBS buffer. After centrifugation (15 min, 800 × g, 4 °C), the pellets were stored at -20 °C. Cells were suspended in lysis buffer (50 mM HEPES (pH 7.4), 200 mM NaCl, 20 mM Imidazole, 5% glycerol, 0.5 mM TCEP) and lysed by sonication on ice (2 min, amplitude 35%). The cell lysates were cleared by centrifugation (60 min, 36,000 × g, 4 °C). The supernatant was combined with Ni Sepharose (GE Healthcare, 7.5 mL) and loaded onto a gravity flow column. After extensive washing with lysis buffer, the TET3_{CD} His10-tagged protein was eluted using the lysis buffer containing 300 mM imidazole. The eluted fractions were further purified using an AKTA Xpress system combined with an S200 gel filtration column (GE Healthcare) equilibrated with the gel filtration buffer (150 mM NaCl, 0.5 mM TCEP, 5% glycerol in 20 mM HEPES (pH 7.4)). The protein yield was 0.6 mg from 3 L of culture, and the purity was confirmed by SDS-PAGE (Figure S1). The purified protein was stored at 0.2 mg mL⁻¹.

TET2_{CDALCI}. A construct encoding for N-terminally His-tagged human TET2 (D1129-G1936 with residues Y1481-N1843 replaced by a 15-residue GS-linker GGGGSGGGGSGGGGS) in the pET-28b vector was kindly provided by the Yanhui Xu laboratory.²⁵ The plasmid was transformed and expressed in *Escherichia coli* BL21(DE3) cells. A 6 × 10 mL overnight culture was used to inoculate 6 L of Terrific Broth media containing kanamycin (100 μg mL⁻¹). Cultures were grown at 37 °C until the OD₆₀₀ reached ~1.0 after which time, the temperature was adjusted to 18 °C; expression culture was induced with 0.5 mM IPTG and the cells were incubated for 18 h. Cells were harvested by centrifugation (5000 rpm, 30 min, 4 °C), suspended in lysis buffer (500 mM NaCl, 20 mM imidazole, 0.5 mM TCEP, 5% (v/v) glycerol with protease inhibitor cocktail (1:2000, EDTA-Free Protease Inhibitor Cocktail, Roche Diagnostics Ltd.) in 50 mM HEPES (pH 7.4), and lysed by sonication at 4 °C. The lysates were cleared by centrifugation (17,000 rpm, 1 h, 4 °C), and loaded onto a Ni NTA column. After extensively rinsing with lysis buffer, the His₆-tagged TET2_{CDALCI} protein was eluted using the lysis buffer containing 300 mM imidazole. The eluted fractions were further purified using an AKTA Xpress system combined with an S200 gel filtration column equilibrated in the gel filtration buffer (150 mM NaCl, 0.5 mM TCEP, and 5% (v/v) glycerol in 20 mM HEPES (pH 7.4)). The elution volume (92 mL) indicated the protein is monomeric in solution. The final yield was ~10 mg of His₆-TET2_{CDALCI} from 6 L culture. The purity was confirmed by SDS-PAGE (Figure S1).

Preparation of Inhibitor Screening Plates for AlphaScreen or SPE-MS Assays. Inhibitors were dry dispensed using an Echo 550 Acoustic dispenser (Labcyte) from inhibitor stock solutions in prepared in DMSO (10 mM). Increments of inhibitor concentrations were made in steps of 2.5 nL and, if necessary, backfilled up to 100 nL (AlphaScreen) or 250 nL (SPE-MS) of total DMSO volume on ProxiPlate (PerkinElmer for AlphaScreen) or PP microplate 384 well (Greiner bio-one, SPE-MS). Intermediate dilutions series were made robotically using a combination of Echo dispensing and MultiDrop (Thermo Scientific, MultiDrop Combi). Every screening plate contained a DMSO control, prequench negative control, and IOX1⁴⁵ as a positive control reference inhibitor. Solutions containing R or S-2HG, succinate, or fumarate are from purified water source solutions with the solution adjusted to a pH of 7.0. Inhibitor plates containing water-based solutions were allowed to dry (ambient conditions) until all water was evaporated prior to conducting the assay.

SPE-MS Assay Configuration. Quenched reaction plates (50 μL per well) were transferred to the RapidFire RF360 and samples

aspirated under vacuum through a sample loop (10 μL, 400 ms) and loaded onto a SPE cartridge (Agilent, Type A C4, G9203-80103). The C4 SPE was washed with solvent A (6 mM octylammonium acetate in LCMS grade water, 4500 ms, 1.5 mL min⁻¹), and the DNA was eluted from the SPE cartridge with solvent B (80% (v/v) acetonitrile in LCMS water, 4500 ms, 1.25 mL min⁻¹) into the Agilent QTOF-6530 mass spectrometer. The SPE cartridge was re-equilibrated (solvent A, 500 ms, 1.25 mL min⁻¹) prior to the next injection. The cartridge was sequentially washed four times with alternating cycles of water and acetonitrile to remove residual DNA and contaminants between each sample injection. Solid octylammonium acetate (Alfa Aesar) was prepared as described.⁸⁵ The mass spectrometer was operated in negative ESI mode with a nebulizer pressure (40 PSIG); gas temperature (350 °C); drying gas flow rate: (9 L min⁻¹); fragmentor voltage (135 V); OCT1 RF Vpp (750 V); and skimmer (65 V).

Cloning of TET1_{CD}, TET2_{CD}, and TET3_{CD}. DNA sequences encoding for the catalytic domains of WT TET1_{CD} (1418–2136 aa), TET2_{CD} (1129–2002 aa), TET3_{CD} (824–1795 aa), and catalytically inactive mutant TET1_{CD}MUT (1418–2136 aa with H1672Y and D1674A) were cloned into pCDNA5-3 × FLAG-FRT/TO and cotransfected with pOG44 Flp-recombinase vector (V600520, Thermo Fisher) in U2OS-TREx cells. Posttransfection (48 h), hygromycin B (100 μg μL) was used for selection; the cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) tetracycline-free FBS (TF-FBS), 2 mM L-glutamine, 10 U/mL penicillin/streptomycin. Single-cell colonies were grown and tested for Dox-inducible expression by Western blot analysis (Figure S7). Successful colonies were expanded and frozen. The cells were tested routinely for mycoplasma contamination.

Synthesis of N-Oxalylglycine Diethylate 38.⁸⁴ A solution of ethyl 2-aminoacetate hydrochloride (HCl·H-Gly-OEt) (333 mg, 2.4 mmol, 1.1 equiv) in CH₂Cl₂ (6.0 mL) under an argon atmosphere was cooled to 0 °C with stirring. Diisopropylethylamine (750 μL, 4.3 mmol, 2.0 equiv) was added, followed by ethyloxalyl chloride (250 μL, 2.2 mmol, 1.0 equiv). The reaction was allowed to warm to room temperature with stirring. After 6 h, the mixture was cooled to 0 °C and quenched by the addition of sat. solution of NH₄Cl (~10 mL). The mixture was extracted with ethyl acetate (3 × 30 mL); the combined organic phases were dried over MgSO₄, filtered and the supernatant was concentrated in vacuo. The crude material was purified by silica gel chromatography using a linear gradient of cyclohexane to ethyl acetate (0–100%) to give a colorless oil (446 mg, 2.2 mmol, 91%). ¹H NMR 400 MHz (CDCl₃) δ (ppm): 7.63 (s, 1 H, NH), 4.31 (q, 2 H, J = 7.5 Hz, CH₂CH₃), 4.19 (q, 2 H, J = 7.5 Hz, CH₂CH₃), 4.07 (d, 2 H, J = 4.5 Hz, NHCH₂), 1.33 (t, 3 H, J = 7.5 Hz, CH₃), 1.24 (t, 3 H, J = 7.5 Hz, CH₃). ¹³C NMR 101 MHz (CDCl₃) δ (ppm): 168.7, 160.0, 157.7, 63.3, 61.9, 41.5, 14.1, and 14.0. The obtained NMR data (Figures S14 and S15) are in agreement with reported values.⁸⁴

Synthesis of NOG Disodium Salt 5.⁸³ To a solution of diethyl NOG (102 mg, 0.50 mmol, 1.0 equiv) in THF/H₂O (3.0 mL, 1:1), NaOH (40 mg, 1.0 mmol, 2.0 equiv) was added; the reaction mixture was stirred overnight at room temperature. Volatiles were removed in vacuo and the crude residue was coevaporated twice with ethanol. The crude material was triturated with a small amount of methanol; the so-obtained precipitate was isolated by filtration to yield a white amorphous solid (77 mg, 81%). ¹H NMR 400 MHz (D₂O) δ (ppm): 3.79 (s, 2 H, CH₂). ¹³C NMR 126 MHz (D₂O) δ (ppm): 176.4, 166.0, 164.8, 43.2.⁸³ ESI-MS as [M + H]⁺, calc: 148.0; observed: 148.1. The obtained NMR data (Figures S16 and S17) are in agreement with reported values.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.3c01820>.

Figures illustrating protein, biochemical and cellular data, details of HPLC and NMR spectra for compounds (PDF)

Molecular formula strings and the associated biochemical and biological data (CSV)

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Author Contributions

R.B. and A.K. conceived the idea and A.K. directed the project. R.B. developed and conducted all enzyme assay experiments. A.T. assisted in the SPE-MS assay development. E.S. produced recombinant TET₂^{CDΔLCI} and TET₃^{CD} while A.S. produced TET₂^{CD} with input from N.B.B. B.B. and H.S. performed the TET1 cellular assays, G.R. cloned TET2/TET3 and H.S. produced TET2/TET3 stable cell lines and TET1–3 cell-based inhibition selectivity assays. S.K. provided input on TET cell-based assays. T.B. and C.J.S. helped supervise the project. R.B. and A.K. wrote the manuscript and all authors contributed to the final version. H.S., E.S., and B.B. contributed equally.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

2OG, 2-oxoglutarate; ^{5m}C, 5-methylcytosine; ^{5hm}C, 5-hydroxymethylcytosine; ^{5f}C, 5-formylcytosine; ^{5ca}C, 5-carboxylcytosine; ^{5ox}C, 5-oxidizedcytosines; Asc, L-ascorbate; BER, Base excision repair; CD, Catalytic domain; CLL, Chronic lymphocytic leukemia; CMML, Chronic myelomonocytic leukemia; D2HGDH, D-2-hydroxyglutarate dehydrogenase; DAPI, 4',6-Diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's Modified Eagle's Medium; DMOG, Dimethylxalylglycine; DNMT, DNA methyl transferase; Dox, Doxycycline; DSBH, Double-stranded β-helix; dsDNA, Double-stranded DNA; *E. coli*, *Escherichia coli*; EMA, European Medicines Agency; FBS, fetal bovine serum; FH, fumerate hydratase; HDAC, histone deacetylases; HEPES, 4-(2-

hydroxyethyl)piperazine-1-ethanesulfonic acid; HIF, hypoxia-inducible factor; IDH, isocitrate dehydrogenase; IF, immunofluorescence; JmjC, JumonjiC; L2HGDH, L-2-hydroxyglutarate dehydrogenase; LCI, low complexity insert; KDM, lysine-specific histone demethylase; MDS, myelodysplastic syndrome; MUT, mutant; NOG, *N*-oxalylglycine; N.D., not determined; N.I., not inhibitory; PHD, prolyl hydroxylase domain; R-2HG, R-2-hydroxyglutarate; S-2HG, S-2-hydroxyglutarate; SDH, succinate dehydrogenase; s.e.m, standard error of the mean; SPE-MS, solid-phase extraction – mass spectrometry; ssDNA, single stranded DNA; TCA, tricarboxylic acid cycle; TDG, thymidine DNA glycosylase; TET, ten-eleven translocation; WT, wild-type

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