nature portfolio

https://doi.org/10.1038/s43018-021-00249-x

Supplementary information

Discovery of a first-in-class reversible DNMT1-selective inhibitor with improved tolerability and efficacy in acute myeloid leukemia

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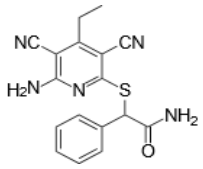
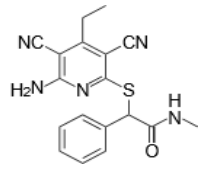
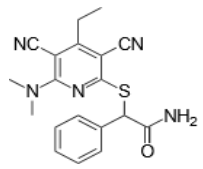
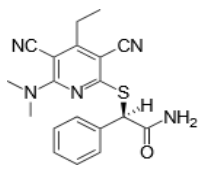
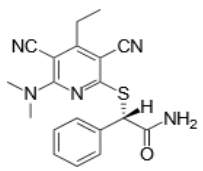
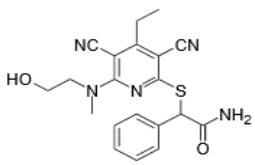
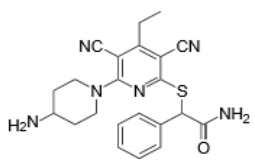
Supplementary Data Table 1. High-throughput screen.

Category	Parameter	Description
Assay	Type of assay	*In vitro* enzymatic scintillation proximity assay (SPA)
	Target	DNMT1 (Gene ID 1786, UniProt P26358)
	Primary measurement	DNMT1 methyltransferase activity measuring the transfer of a [^{3}H]-methyl group from ^{3}H-SAM to a cytidine contained within a hemi-methylated DNA substrate. Inhibition of DNMT1 was measured as a decrease in signal.
	Key reagents	DNMT1 (601-1600, Proteros), ^{3}H-SAM (American Radiolabeled Chemicals Inc), 40-mer hemi-methylated DNA duplex (IDT), PEI PS Imaging beads (PerkinElmer)
	Assay protocol	See Methods: High-throughput screen
	Additional comments	Radioactive assay
Library	Library size	1,842,902
	Library composition	Small molecule
	Source	GlaxoSmithKline
Screen	Format	1536 well (Greiner 782075)
	Concentration(s) tested	Final: 10 µM compound, 1% DMSO
	Plate controls	High: no compound; Low: no enzyme
	Reagent/ compound dispensing system	Reagents: Multidrop Combi (Thermo Scientific); Compound: Echo Acoustic Dispenser (Labcyte)
	Detection instrument and software	Viewlux (PerkinElmer)
	Assay validation/QC	Mean z' = 0.69 (1349/1354 plates passed)
	Correction factors	None
	Normalization	Raw data normalization to % inhibition (relative to control wells)
Post-HTS analysis	Hit criteria	≥30% Inhibition
	Hit rate	0.44% (8034/1842902)
	Additional assay(s)	Single-shot confirmation and IC_{50} determination in primary assay. Non-specific DNA binding, DNMT1 (FL) fluorescence-coupled breaklight assay.
	Confirmation of hit purity and structure	Compounds were resynthesized
	Additional comments	The majority of hits were subsequently filtered for nuisance liabilities such as array chemistry (typically inactive after purification) and IFI (inhibitory frequency index).

Supplementary Data Table 2. Structure-Activity Relationship (SAR).

Compound Number	Structure	DNMT1 IC_{50} (μM)[a]	chromLogD pH7.4[b]	FaSSIF[c] (μg/mL)
GW623415X HTS hit		2.78 ± 0.25 (n=26)	4.1	5
GSK3510477		>10 (n=6)[d]	4.5	6
GSK3482364		0.43 ± 0.25 (n=22)	4.9	< 1.6
GSK3484862 (*R*)-enantiomer		0.23 ± 0.02 (n=34)	4.9	< 1
GSK3484861 (*S*)-enantiomer		>10 (n=6)[d]	4.9	1
GSK3730808		1.81 ± 0.11 (n=4)	4.0	7.4
GSK3685032		0.17 ± 0.02 (n=12)	2.7	165

[a] Fluorescence-coupled breaklight assay; n = biologically independent replicates; average ± SEM

[b] Measure of compound lipophilicity

[c] Measure of compound solubility

[d] Value adjusted based on solubility, experimentally IC_{50} > top dose tested (200-500 μM)

Supplemental Data Table 3. GSK3685032 inhibition of a methyltransferase panel.

Assay Format	Enzyme	Substrate	GSK3685032 IC_{50} (μM) ± SEM		SAH[a] IC_{50} (μM) ± SEM
Radioactive Scintillation Proximity Assay	DNMT1	DNA duplex	0.036 ± 0.001 (n=70)		0.54 ± 0.02 (n=41)
	DNMT3A/3L	DNA duplex	> 100 (n=8)		0.10 ± 0.01 (n=10)
	DNMT3B/3L	DNA duplex	> 100 (n=8)		0.09 ± 0.01 (n=10)
Assay Format	**Enzyme**	**Substrate**	**IC_{50} (μM)**	**% Inhibition[b]**	**IC_{50} (μM)**
Radioactive HotSpot Technology	ASH1L	Nucleosomes	> 10	-2	ND
	DOT1L	Nucleosomes	> 10	1	0.3
	EZH1 Complex	Core Histone	> 10	15	13.9
	EZH2 Complex	Core Histone	> 10	-4	40.8
	G9a	Histone H3 (1-21)	> 10	10	1.2
	GLP	Histone H3 (1-21)	> 10	12	1.2
	METTL21A	HSPA8-[CTD]	> 10	16	51.5
	MLL1 Complex	Nucleosomes	> 10	17	0.3
	MLL2 Complex	Nucleosomes	> 10	7	10.0
	MLL3 Complex	Core Histone	> 10	-6	4.3
	MLL4 Complex	Nucleosomes	> 10	17	0.8
	NRMT1	RCC1	> 10	9	0.3
	NRMT2	RCC1	> 10	0	0.7
	NSD1	Nucleosomes	> 10	11	2.4
	NSD2	Nucleosomes	> 10	9	2.4
	NSD3	Nucleosomes	> 10	16	ND
	PRDM9	Histone H3	> 10	-25	ND
	PRMT1	Histone H4	> 10	4	0.1
	PRMT3	Histone H4	> 10	7	0.5
	PRMT4	Histone H3	> 10	-5	0.1
	PRMT5/MEP50	Histone H2A	> 10	10	0.6
	PRMT6	GST-GAR	> 10	-2	0.2
	PRMT7	GST-GAR	> 10	6	0.0
	PRMT8	Histone H4	> 10	16	0.1
	SET1b Complex	Core Histone	> 10	34	3.1
	SET7/9	Core Histone	> 10	9	58.3
	SET8	Nucleosomes	> 10	12	ND
	SETD2	Nucleosomes	> 10	-10	1.9
	SMYD2	Histone H4	> 10	0	ND
	SMYD3	MEKK2	> 10	7	31.1
	SUV39H1	Histone H3	> 10	-30	104.9
	SUV39H2	Histone H3	> 10	-6	39.9
	SUV420H1TV2	Nucleosomes	> 10	8	70.9
	S-COMT	RBC-DA1	> 10	1	ND

[a] The product inhibitor, SAH, was included as a control inhibitor.

[b] Inhibition (%) values listed are at 10 μM GSK3685032.

N=1 biological replicate unless otherwise noted.

Supplementary Data Table 4. Summary of X-ray data collection at wavelength=1Å (APS-SERCAT-22ID) and refinement statistics in space group *C*2

Inhibitor	None	GSK3685032	GSK3830052
Date of data collection	10/2018	06/2019	10/2019
PDB Code	6X9I	6X9K	6X9J
Cell dimensions (Å)	161.89, 77.70, 115.41	161.32, 78.34, 117.07	160.06, 77.77, 116.72
α=γ=90°, β (°)	125.7	125.8	125.5
Resolution (Å)	45.21-2.20 (2.33-2.20)*	40.00-2.65 (2.74-2.65)	34.92-1.79 (1.85-1.79)
[a] R_{merge}	0.159 (0.821)	0.132 (0.749)	0.089 (0.739)
R_{pim}	0.076 (0.662)	0.048 (0.385)	0.036 (0.686)
$CC_{1/2}$, CC	(0.453, 0.789)	(0.545, 0.840)	(0.365, 0.731)
[b] <I/σI>	9.4 (1.6)	14.8 (1.7)	19.6 (0.9)
Completeness (%)	95.3 (81.5)	98.6 (90.3)	94.5 (72.9)
Redundancy	4.3 (2.4)	7.9 (4.0)	6.7 (4.1)
Observed reflections	236,316	269,959	690,353
Unique reflections	55,268 (4,682)	34,128 (3,087)	103,653 (7,967)
Refinement			
Resolution (Å)	2.20	2.69	1.79
No. reflections	55,112	34,113	103,497
[c] R_{work} / [d] R_{free}	0.194 / 0.225	0.182 / 0.228	0.200 / 0.229
No. Atoms			
Protein	6194	6553	6518
DNA	487	487	487
SAH	26	-	-
Inhibitor	-	30	32
Zn	2	2	2
Solvent	247	225	458
B Factors (Å^2)			
Protein	70.2	67.4	53.2
DNA	90.7	107.6	109.9
SAH	41.1	-	-
Inhibitor	-	74.3	65.7
Zn	90.8	82.1	54.9
Solvent	56.9	56.6	49.9
R.m.s. deviations			
Bond lengths (Å)	0.004	0.004	0.004
Bond angles (°)	0.7	0.7	0.7

* Values in parenthesis correspond to highest resolution shell.

[a] R_{merge} = Σ | I - <I>| /Σ I, where I is the observed intensity and <I> is the averaged intensity from multiple observations.

[b] <I/σI> = averaged ratio of the intensity (I) to the error of the intensity (σI).

[c] R_{work} = Σ | Fobs - Fcal | /Σ | Fobs |, where Fobs and Fcal are the observed and calculated structure factors, respectively.

[d] R_{free} was calculated using a randomly chosen subset (5%) of the reflections not used in refinement.

Supplementary Data Table 5. Analytical compound characterization

Compound Number	LCMS[a] (m/z)	^{1}H NMR[b]	Analytical HPLC[c] (purity)	HRMS[d] (m/z)
GSK3510477	352 $[M+H]^+$	^{1}H NMR (300 MHz, DMSO-d6) δ ppm 8.23 (d, *J* = 4.7 Hz, 1H), 7.91 (s, 2H), 7.63 – 7.53 (m, 2H), 7.41 – 7.24 (m, 3H), 5.55 (s, 1H), 2.69 (q, *J* = 7.6 Hz, 2H), 2.59 (d, *J* = 4.6 Hz, 3H), 1.18 (t, *J* = 7.5 Hz, 3H)	96.8% (Rt 7.1 min, 254 nm)	$[M+H]^+$ calcd for $C_{18}H_{17}N_5OS$, 352.1232; found, 352.1229
GSK3482364	365.9 $[M+H]^+$	^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.47 – 7.43 (m, 2H), 7.42 – 7.34 (m, 3H), 6.55 (br s, 1H), 5.60 (br s, 1H), 5.43 (s, 1H), 3.40 (s, 6H), 2.92 (q, *J* = 7.6 Hz, 2H), 1.32 (t, *J* = 7.6 Hz, 3H)	99.7% (Rt 4.2 min, 254 nm)	$[M+H]^+$ calcd for $C_{19}H_{19}N_5OS$, 366.1389; found, 366.1398
GSK3484862 (*R*)-enantiomer	366.1 $[M+H]^+$	^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.92 (s, 1H), 7.55 – 7.47 (m, 2H), 7.42 – 7.28 (m, 4H), 5.58 (s, 1H), 3.34 (s, 6H), 2.75 (q, *J* = 7.6 Hz, 2H), 1.19 (t, *J* = 7.6 Hz, 3H)	chiral purity > 99.8% ee (Rt 3.871 min, 270 nm); optical rotation: $[\alpha]^{D}_{24}$ (deg cm^3 g^{-1} dm^{-1}) = -337 (c=0.2, chloroform)	$[M+H]^+$ calcd for $C_{19}H_{19}N_5OS$, 366.1389; found, 366.1394
GSK3484861 (*S*)-enantiomer	366.2 $[M+H]^+$	^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.92 (s, 1H), 7.55 – 7.48 (m, 2H), 7.41 – 7.30 (m, 4H), 5.58 (s, 1H), 3.34 (s, 6H), 2.75 (q, *J* = 7.6 Hz, 2H), 1.19 (t, *J* = 7.6 Hz, 3H)	chiral purity = 99.8% ee (Rt 6.206 min, 270 nm); optical rotation: $[\alpha]^{D}_{24}$ (deg cm^3 g^{-1} dm^{-1}) = +339 (c=0.2, chloroform)	N.D.
GSK3730808	396.1 $[M+H]^+$	^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.90 (s, 1H), 7.52 – 7.47 (m, 2H), 7.41 – 7.30 (m, 4H), 5.52 (s, 1H), 4.84 (t, *J* = 5.6 Hz, 1H), 3.87 (qt, *J* = 14.6, 5.4 Hz, 2H), 3.70 – 3.58 (m, 2H), 3.39 (s, 3H), 2.75 (q, *J* = 7.6 Hz, 2H), 1.20 (t, *J* = 7.6 Hz, 3H)	99.7% (Rt 6.78 min, 254 nm)	N.D.
GSK3685032	421.1 $[M+H]^+$	^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.94 (s, 1H), 7.54-7.50 (m, 2H), 7.41-7.31 (m, 4H), 5.53 (s, 1H), 4.41 (d, *J* = 13.4 Hz, 2H), 3.31-3.25 (m, 2H), 2.96-2.87 (m, 1H), 2.75 (q, *J* = 7.6 Hz, 2H), 1.84 (d, *J* = 12.4 Hz, 2H), 1.38-1.24 (m, 2H), 1.20 (t, *J* = 7.6 Hz, 3H) (2H obscured by water)	100% (Rt 4.9 min, 254 nm)	$[M+H]^+$ calcd for $C_{22}H_{24}N_6OS$, 421.1811; found, 421.1809
GSK3830052	473.2 $[M+H]^+$	^{1}H NMR (400 MHz, MeOH-d_4) δ ppm 7.49 (d, *J* = 8.6 Hz, 2H), 7.43 (d, *J* = 8.6 Hz, 2H), 4.58 (s, 2H), 4.04 (t, *J* = 6.6 Hz, 2H), 3.50 (s, 3H), 3.32 (s, 3H), 3.24 (t, *J* = 6.6 Hz, 2H), 2.99 – 2.90 (m, 5H), 2.67 (s, 3H), 1.33 (t, *J* = 7.6 Hz, 3H)	99.9% (Rt 3.77 min, 254 nm)	N.D.
GSK3844831	551.8 $[M+H]^+$	^{1}H NMR (400 MHz, MeOH-d_4) δ ppm 7.75-7.90 (m, 4H), 7.64-7.71 (m, 3H), 7.52-7.58 (m, 2H), 5.62 (s, 1H), 3.83-4.04 (m, 4H), 3.70 (t, *J* = 6.0 Hz, 2H), 2.79-3.10 (m, 8H), 2.08 (br. s, 2H), 1.32 (t, *J* = 7.6 Hz, 3H)	98.9% (Rt 3.55 min, 254 nm)	$[M+H]^+$ calcd for $C_{31}H_{33}N_7O_3$, 552.2723; found, 552.2724
GSK3901839	556.2 $[M+H]^+$	^{1}H NMR (400 MHz, MeOH-d_4) δ ppm 7.62 (d, *J* = 8.4 Hz, 2H), 7.30 (d, *J* = 8.1 Hz, 2H), 5.55 (s, 1H), 3.77-3.94 (m, 4H), 3.65 (t, *J* = 6.0 Hz, 2H), 2.80-2.90 (m, 3H), 2.57-2.76 (m, 5H), 1.80-2.04 (m, 2H), 1.30 (t, *J* = 7.6 Hz, 3H)	100% (Rt 2.84 min, 254 nm)	$[M+H]^+$ calcd for $C_{26}H_{28}F_3N_9O_2$, 556.2396; found, 556.2402

[a] LCMS analysis was performed on a PE Sciex Single Quadrupole 150EX or Waters Acquity SQD UPLC/MS system, using a Thermo Hypersil Gold (C18, 20 × 2.1 mm, 1.9 µm particle diameter), 4−95% CH_3CN/H_2O (with 0.02% TFA) over 2 min, flow rate = 1.4 mL/min at 55 °C. m/z = mass-to-charge ratio.

[b] ^{1}H NMR spectra were recorded on a Bruker Advance or Varian Unity at 300 or 400 MHz as solutions in DMSO-d_6 unless otherwise stated. Chemical shifts (δ) are reported in ppm relative to an internal solvent reference. Apparent peak multiplicities are described as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), or m (multiplet). Coupling constants (*J*) are reported in hertz (Hz) before the integration.

[c] Analytical HPLC was performed on an Agilent 1100 series system using a Zorbax SB-C8 column (4.6 mm x 150 mm, 5 µm), eluting with 5−100% CH_3CN/H_2O (with 0.02% TFA) over 12.5 min followed by a hold for 2.5 min at a flow rate of 1.5 mL/min at 40 °C. The retention time (Rt) is expressed in minutes at a UV detection of 254 or 270 nm. Chiral purity was performed using a Lux-2 cellulose column (4.6 mm x 150 mm, 5 µm), eluting with 100:0.1 methanol:isopropylamine (isocratic) at a flow rate of 1.0 mL/min and UV detection of 270 nm.

[d] High resolution mass spectrometry (HRMS) was performed by either a time-of-flight mass spectrometer or Fourier transform mass spectrometer using electrospray (ES) techniques. m/z = mass-to-charge ratio. N.D. = not determined.