Design and synthesis of pyridone containing 3,4-dihydroisoquinoline-1(2H)-ones as a novel class of EZH2 inhibitors

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Supplementary Data

Content of Supporting Information:

I. Methods for enzyme and oligonucleosome purification

EZH2 four protein complex:

Expression and purification of the EZH2/EED/SUZ12/RBBP4 protein complex: The proteins were expressed in SF9 cells by con-infection of the baculoviruses carrying the expression constructs for EED, EZH2, SUZ12 and RBBP4. EED is FLAG tagged at the N-terminal. The proteins are purified with FLAG affinity chromatography followed by size exclusion chromatography.

Oligonucleosomes:

Hela cell nuclear extract was digested with micrococcal nuclease. Sucrose gradient containing oligo nucleosomes were collected.

II. Enzyme Assay

EZH2 enzymatic activity was monitored by following the incorporation of a radioactive methyl group from the co-substrate SAM into oligonucleosomes isolated from HeLa cells[a,b]. PRC2 four protein complex (EZH2, SUZ12, EED, RbAp48) containing either wild-type or Y641N mutant EZH2, 5 nM, was diluted into assay buffer (100 mM Tris pH 8.5, 4 mM DTT and 0.01% Tween-20) containing 25 ug/ml oligonucleosomes. The reaction was initiated with the addition of 1.5 mM ^{3}H SAM (0.94 uCi/well) and allowed to proceed for 60 minutes. Following addition of 2x the reaction volume of 20% TCA, the reaction mixture was filtered through Multiscreen PVDF filter plates (Millipore, MSIPN4B10), washed 5x with PBS and dried. Activity was measured by scintillation counting. IC50 values were generated by Pfizer proprietary software by fitting to the standard 4-parameter IC50 equation. For K_i determination, the assay was run as described for IC50 determination except the SAM concentration was 15 μM. The data were fit to the Morrison equation for tight binding competitive inhibition. For mechanistic studies, the data were fit to models for competitive, non-competitive and mixed inhibition using Graphpad Prism software with competitive inhibition being the best model to fit the data. While the competitive inhibition model is clearly preferred, we cannot completely rule out a mixed competitive model.

Reference:

a) Schnitzler, G.R. Isolation of Histones and Nucleosome Cores from Mammalian Cells *Current Protocols in Molecular Biology* **2000** Supplement 50, 21.5.1-21.5.12.
b) Fang J.; Wang H.; Zhang Y. Purification of histone methyltransferases from HeLa cells. *Methods Enzymol.* **2003**, *377*, 213-226.

III. Computational Methods

Gas-Phase and PB continuum solvent DFT calculations were performed using the program JAGUAR (a) at the M06-2X/cc-pVTZ(-f) level of theory as this was shown to well-reproduce CCSD(T)/CBS conformer and rotamer energetics (b).

All searches of the Cambridge Crystallographic Database (CCD) were performed using Conquest V1.17 (c). Torsion angle data that resulted from the database queries was binned in increments of 10 degrees from 0 to 180 degrees with negative values being mapped onto positive values as a result of molecular symmetry. Each bin was then converted to a percentage of the total sample. Statistical Analyses were performed using JMP version 10.0.2 [d]. A stepwise, multiple linear regression analysis was completed for inhibitors (1,3,6-9) for the purpose of identifying a model that related the binned percentages to the log IC50. A single, statistically significant binned torsion angle descriptor emerged from this analysis.

Conformational entropy calculations were performed using the Monte Carlo Multiple Minimum method as implemented in Macromodel [e] according to a procedure previously reported [f]. The conformational searches employed the OPLS3 force field along with the GB/SA continuum water model and a maximum of 25000 Monte Carlo Steps.

a) Jaguar, v8.8; Schrodinger, Inc., New York, NY, 2015.
b) Bochevarov, A.D.; Harder, E.; Hughes,T.F.; Greenwood, J.R.; Braden, D.A.; Philipp, D.M.; Rinaldo, D.; Halls, M.D.; Jing Zhang, J.; Friesner, R.A. "Jaguar: A High Performance Quantum Chemistry Software Program with Strengths in Life and Materials Sciences" Int. J. Quantum Chem. 2013, 113, 2110–2142.
c) Conquest v1.17, csd v5.36 (nov 2014). computer program and database. CCDC 2014
d) JMP, 1989–2012. Version 10.0.2. computer program. SAS Institute Inc.
e) Macromodel v10.8; Schrodinger, Inc., New York, NY, 2015.
f) Guimarães, C. R.W.; Cardozo, M. MM-GB/SA Rescoring of Docking Poses in Structure-Based Lead Optimzation. *J. Chem. Inf. Model.* **2008**, *48*, 958-970.

IV. Isothermal titration calorimetry experiment

ITC experiments were carried out on a Microcal VP ITC instrument (Malvern Instruments) at 20 ^{o}C. Protein samples were exchanged into a buffer containing 150 mM NaCl, 25 mM HEPES, pH 8.0, 10% (v/v) glycerol, 1mM TCEP, by dialysis and the concentration determined spectrophotometrically using an E_{280} of 298,400 M^{-1} cm^{-1} for the WT 4-protein PRC2 complex (LJIC-787). In a typical experiment, 19 x 15 μL injections of 33 μM compound were made into 3.3 μM PRC2 complex. Heats of dilution were determined from the saturation end point. Data from the direct binding experiments were analyzed using the ORIGIN software (OriginLab) and fit to a simple 1:1

equilibrium binding model (a). Standard deviation was calculated from repeat experiments in triplicate.

References

a) Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) Rapid measurement of binding constants and heats of binding using a new titration calorimeter, *Analytical biochemistry 179*, 131-137.

V. In vitro cell and tumor sample-H3K27Me3 and gene analysis

RNA Isolation

For in-vitro samples, cells were harvested following treatment and RNA was prepared using Qiagen QIAshredder (#79654) and RNeasy (#74104) kits and standard protocols. In-vivo samples were frozen in liquid nitrogen immediately after harvesting after specified days (Day 11 and Day 21) of treatment, and stored in 2ml centrifuge tubes. One 5-mm stainless steel bead (Qiagen, #69989) and 1ml QIAzol Lysis Reagent (Qiagen, #79306) were added to each tube and lysed on the TissueLyser II (Qiagen, #85300) 2x24 micro-centrifuge assembly for 5 min at max speed. The samples were spun down (14,000 rpm, 4° C, 10min), the pulverized aqueous sample (~900µL) transferred to a fresh micro-centrifuge tube, and 400µL of chloroform was added. The samples were vortexed and stored overnight at 4° C. The following day, the samples were vortexed again, spun down (14,000 rpm, 4° C, 10 min), and the top clear, aqueous layer (~700 µL) was removed and processed according to the Qiagen RNeasy kit protocol.

qRT-PCR

One-step qRT-PCR was performed using ThermoFisher Scientific SuperScript III Platinum One-Step qRT-PCR Kit (#11732-088) on Applied Biosystems ViiA 7 real-time cycler. TaqMan gene expression assays were supplied by Applied Biosystems; normalizer: RPN1 (Hs00161446_m1), genes of interest: PRDM1 (Hs00153357_m1), TNFRSF21 (Hs01560899_m1). Experiments were performed in triplicate and means and SD were plotted using GraphPad Prism software.

Histone Extraction

Histone extraction was performed using the EpiQuick Histone Extraction Kit (Epigentek OP0006). Frozen tumor samples were homogenized in pre-lysis buffer using a TissueLyser (Qiagen). The homogenized mixture was transferred to a 15 mL tube and spun down at 3,000 rpm for 5 minutes at 4° C. The tissue pellet was resuspended in 3 volumes (approximately 200ul per 100mg of tissue) of lysis buffer, incubated on ice for 30 minutes, and spun down at 12,000 rpm for 5 minutes at 4° C. The supernatant (containing acid soluble proteins) was transferred to a new tube, and 0.3 volumes of Balance-DTT buffer was immediately added. Extracts were aliquoted and stored at -20° C (short term) or -80° C (long term). Protein concentration was quantified using the DC Protein Assay Kit (Bio-Rad 500-0116).

H3K27Me3 ELISA Assay

Histone extracts were diluted to a final concentration of 400ng in 100ul of coating buffer (PBS containing 0.05% BSA). Each sample was added in triplicate to a 96-well assay microplate (Corning Costar), sealed tightly, and incubated overnight at 4° C. The following day, the wells in each plate were washed three times with 300ul wash buffer (PBS, 0.05% Tween 20) and then blocked with 300ul of blocking buffer (PBS, 0.05% Tween 20, 2% BSA) for 2 hours at room temperature. Following another round of washing, 100ul of detection antibody (Cell Signaling 9733 H3K27me3 diluted 1:2000 in blocking buffer; ABCAM ab1791 total histone H3 diluted 1:5000 in blocking buffer) was added to each well and incubated at room temperature for 1.5 hours. Following another round of washes, 100ul of secondary antibody (anti-Rb-IgG-HRP, Cell Signaling 7074) diluted 1:2000 (H3K27me3) or 1:10000 (total H3) was added to each well and the plate was incubated at room temperature for 1.5 hours. Following another round of washes, detection was carried out by adding 100ul of TMB Super Sensitive Substrate (BioFx Laboratories) to each well, incubating the plate for 10 minutes, adding 100ul of Stop Solution to each well, shaking gently, and reading absorbance at 450 nm.

Western Blot Analysis

Western blot analysis was performed using the Novex NuPAGE SDS-PAGE gel system (ThermoFisher Scientific). Purified histone extracts containing LDS buffer and reducing agent were separated on 4-12% Bis-Tris, transferred to 0.45uM nitrocellulose

membranes, and probed with antibodies to total histone H3 (Cell Signaling 9715) and H3K27me3 (Cell Signaling 9733).

VI. Tables and Figures

Table 1S.

Global minimum energy DFT gas-phase and continuum solvent phase M06-2X/cc-pVTZ(-f) optimized geometric parameters for the CCC=O Torsion Angle.

Compound Number	Gas-Phase Optimized CCC=O Angle	Continuum Water-Phase Optimized CCC=O Angle
10	38.1	51.4
14	48.3	62.7
16	55.0	57.0
17	50.3	52.2
18	22.6	27.1
21	16.7	23.3

Figure 1S.

IC_{50} plotted as a function of the ratio of SAM concentration to SAM K_m. The linear increase in IC_{50} is consistent with a SAM competitive mechanism.

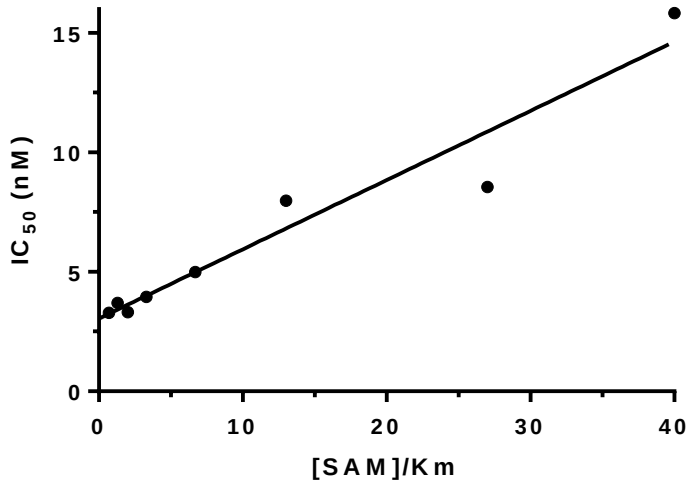


Table 2S.

HMT selectivity data

Enzyme	% Inhibition	SD
EZH2	97.6	1.2
EZH1	99.1	3.4
Dot1L	9.7	8.6
EHMT1	-2.1	2.8
G9a	-8.4	1.2
MLL complex	4.6	11
PRMT1	4.8	2.2
PRMT3	-7.5	3.8
PRMT4	-11.0	0.47
PRMT5/MEP50	1.0	4.2
PRMT6	-11.5	2.0
SETD7	-18.4	2.5
SETD8	-9.8	18.9
SETDB1	6.4	0.36
Suv39H2	-4.7	2.5
Suv420H2	-9.3	6.7

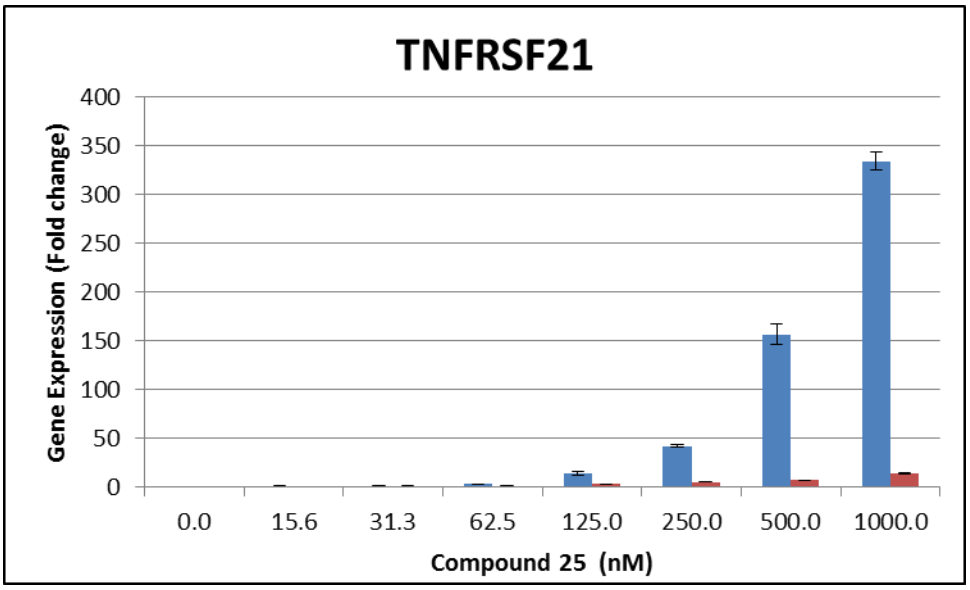
KSS selectivity data

Enzyme	% inhibition	SD
ABL1	2.5	0.8
AKT1	3.7	2.1
BTK	-4.8	4.0
CAMK2A	8.3	2.2
CDK2	9.2	1.5
CHEK1	-4.9	1.2
CHEK2	1.2	2.8
CSNK1A1	4.1	2.5
CSNK2A2	1.8	0.5
EGFR	-5.7	0.1
EPHA2	-2.6	3.1
FGFR1	5.9	8.9
GSK3B	10.4	0.7
INSR	-4.5	1.1
IRAK4	-10.0	13.4
JAK3	9.4	0.1

KDR	**7.8**	**15.1**
LCK	**3.6**	**1.3**
MAP4K4	**29.5**	**2.9**
MAPK1	**3.9**	**3.5**
MAPKAPK2	**14.4**	**3.3**
MARK1	**9.2**	**2.7**
MET	**-14.6**	**8.5**
MST4	**13.2**	**2.3**
MYLK2	**-0.2**	**11.4**
NTRK1	**8.3**	**4.8**
p38	**6.3**	**1.6**
PAK4	**7.1**	**0.3**
PDK1	**1.0**	**2.3**
PIM2	**2.2**	**1.0**
PRKACA	**1.3**	**0.2**
PRKCB2	**2.7**	**1.7**
ROCK1	**7.2**	**1.2**
SGK	**3.1**	**0.8**
SRC	**5.2**	**1.8**
STK3	**3.9**	**0.7**
STK6	**12.0**	**6.4**
TAOK2	**-4.0**	**2.4**
TEK	**9.2**	**0.1**
TGFBR2	**5.9**	**9.8**

Figure 2S.

KARPAS-422 (EZH2 Y641N)
OCI-LY19 (EZH2 wild-type)



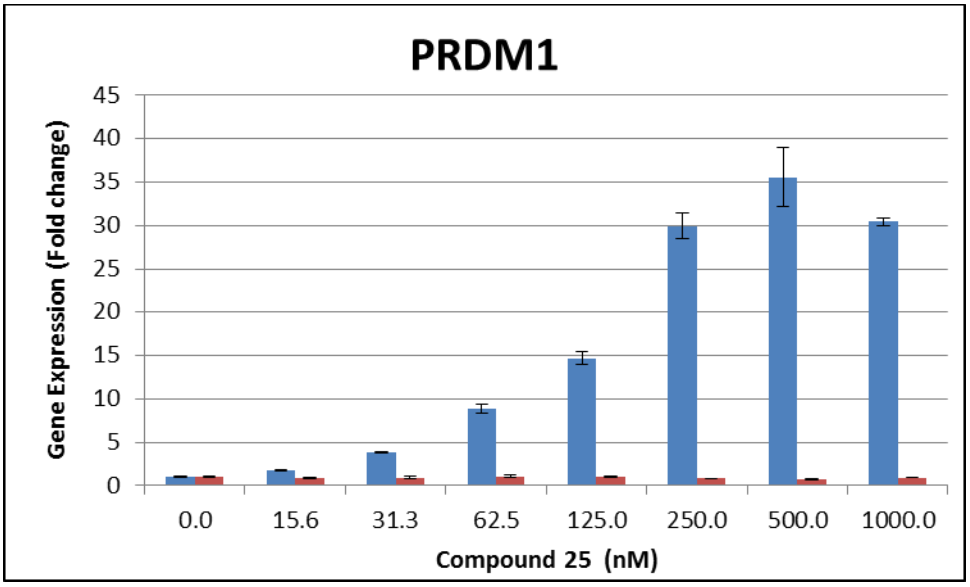
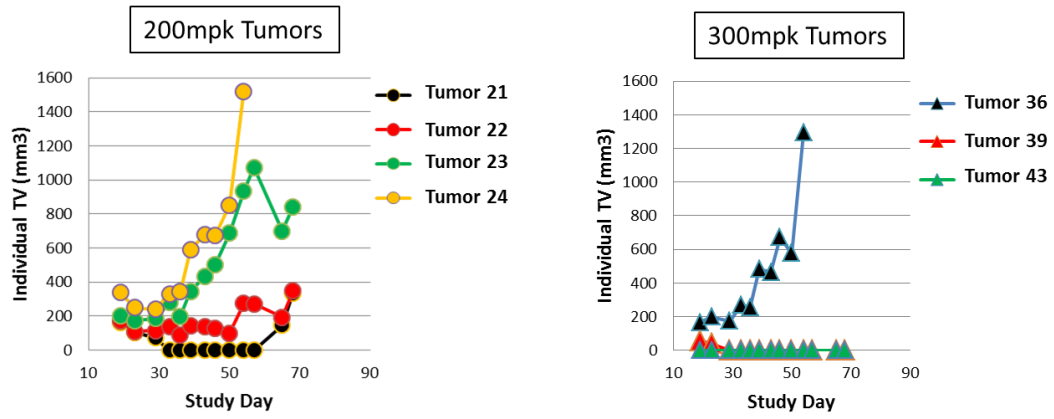


Figure 3S.

A.



B.

C.