EZH2 inhibitors abrogate upregulation of trimethylation of H3K27 by CDK9 inhibitors and potentiate its activity against diffuse large B-cell lymphoma

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Supplementary Material

Supplementary Methods

Chemicals and antibodies

CDKI-73 was kindly provided by Professor Shudong Wang (University of South Australia, Adelaide, Australia). Flavopiridol HCl (S2679), EZP6438 (S7128), GSK126 (S7061), SNS-032 (S1145), AT7519 (S1524), and Dinaciclib (S2768) were purchased from Selleck Chemicals (Houston, TX, USA). The primary antibodies: p-RNA Pol II ser2 (ab70324), UTX (ab84190) (Abcam, Cambridge, UK); PARP (9542S), XIAP (2042S), MCL1 (4572S), β-Tubulin (2146S), BCL-XL (2764S), GAPDH (5174S), H3K79me3 (4260S), H3K36me3 (9763S), H3K9me2 (4658S), H3K27me1 (7693S), H3K27me2 (9728S), H3K27me3 (9733S), Histone H3 (4499S), β-Actin (4970S), EZH2 (5246S), JMJD3 (3457S), CDK9 (2316S), γ-H2AX (9718S) (Cell Signaling Technology, MA, USA); RNA Pol II (sc-56767), SUZ12 (sc-271325), EED (sc-293203) (Santa Cruz Biotechnology, CA, USA).

Cell culture

The lymphoma cells Pfeiffer, SU-DHL-6, breast cancer cells MCF-7, colorectal cancer cells SW620, gastric cancer cells SGC-7901, and embryonic kidney cells HEK293T were purchased from ATCC (Manassas, VA, USA). The lymphoma cells Karpas-299, HT, RL, DB, SU-DHL-5, Will-1, Will-2, U2932, Karpas-1106P, Karpas-422, SU-DHL-4, SU-DHL-8, WSU-DLCL2, and breast cancer cells MDA-MB-453 were purchased from DSMZ (Brunswick, Germany). All these cells were maintained following the providers' instructions.

Proliferation and apoptosis assays

The cytotoxicity of CDKI-73 alone and the combination treatment were investigated using a panel of human DLBCL cell lines and solid tumor cells. Cells plated in 96-well plates were treated with gradient concentrations of the compounds at 37℃ for appropriate time. A cell proliferation assay was carried out using resazurin (Sigma Aldrich, Darmstadt, Germany) for DLBCL cells and

Sulforhodamine B (Sigma Aldrich) for solid tumor cells. The percent of cells in apoptosis was determined using Annexin V-FITC/PI double staining kit (Vazyme Biotech, Nanjing, China) and analyzed using BD FlowJo software.

Comet assay

Slides were pre-coated with 1% normal melting point agarose. About 3×10^4 cells were mixed with 70 μl of 1% low melting point agarose in phosphate-buffered saline (PBS), and rapidly spread onto the pre-coated slides. The slides were immediately placed in cold lysis buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 7.5), 1% Triton X-100, 1% INCI, and 10% DMSO at 4℃ for 1–3 h. The slides were then placed in the electrophoresis solution for 20 min to facilitate DNA unwinding before electrophoresis was conducted for 20 min at 25 V and 300 mA. After electrophoresis, the slides were washed with PBS and then stained with DAPI. The individual cells were viewed using an Olympus BX51 UV florescence microscope (Olympus, Japan).

Colony formation assay

Cells were seeded into 6-well plates at a concentration of 500- 1000 cells per well. After 24 h, cells were treated with indicated compounds for about 2 weeks. Colonies were fixed with fixation solution (10% methanol + 10% acetic acid) at room temperature for 15 min and then stained with a solution of 1% crystal violet in methanol for 15 min.

RNA isolation and Real-Time PCR analysis

RNA from cell lines was isolated using Trizol extraction (Thermo Fisher Scientific). cDNA was prepared using HiScript II 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme Biotech). RT-qPCR was performed following the instruction for ChamQ SYBR qPCR Master Mix (Vazyme Biotech) with V7 Real-Time PCR system (Thermo Fisher Scientific). Expressed values relative to control were calculated using the DDCT method. β-actin was used as a housekeeping gene for

normalization. Results were represented as fold expression. The sequences of primers used for qPCR analysis were listed below.

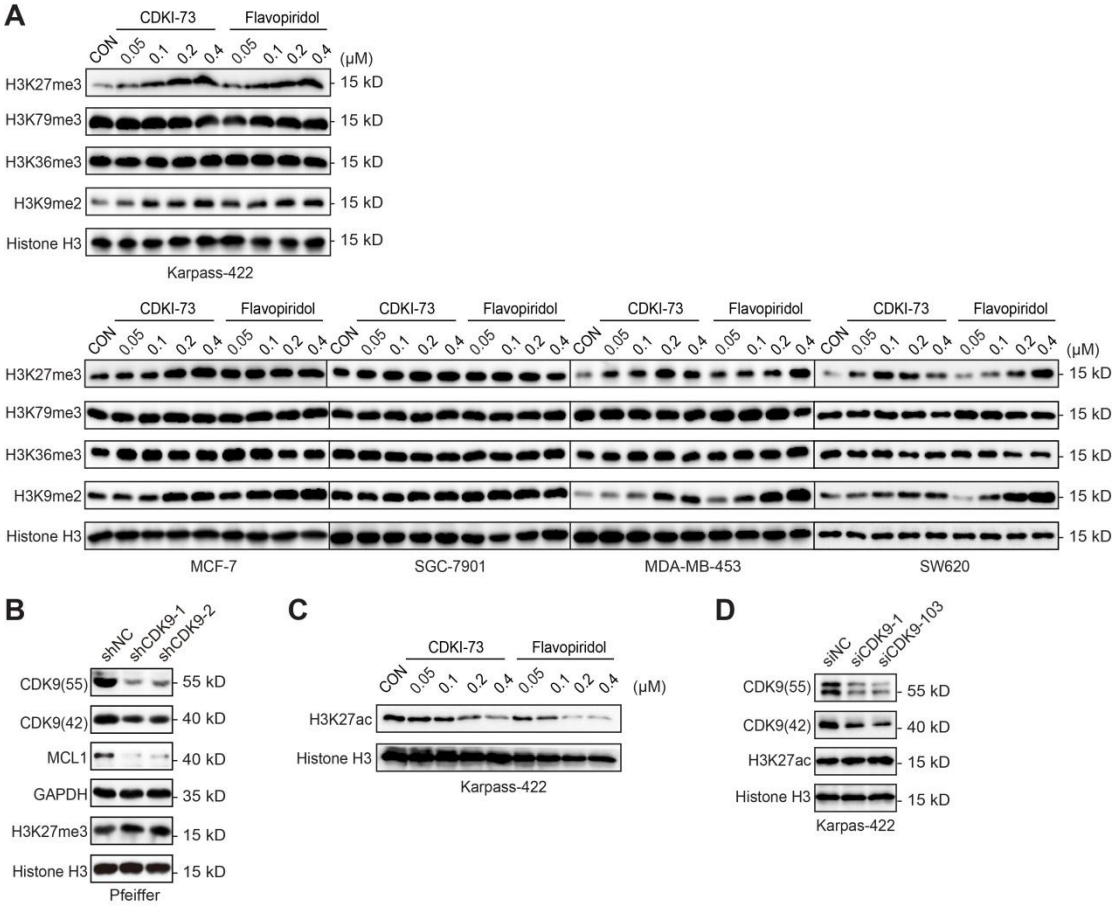
Gene	Forward primer sequence	Reverse primer sequence
Mcl-1	5’-AAAAGCAAGTGGCAAGAGGA-3’	5’-TTAATGAATTCGGCGGGTAA-3’
XIAP	5’-TGGCATTTCCAGATTGGGGC-3’	5’-TAGGACTTGTCCACCTTTTCGC-3
GAPDH	5’-GAAGGTGAAGGTCGGAGT-3’	5’-GAAGATGGTGATGGGATTTC-3’
β-Actin	5’-GCGAGAAGATGACCCAGATC-3’	5’-GGATAGCACAGCCTGGATAG-3’
GATA4	5’-GCGGAAAGAGGGGATCCAAA-3’	5’-CTTGTGGGGAGAGCTTCAGG-3’
CDKN2A	5’-CGACTCTGGAGGACGAAGTT-3’	5’-CCAGGAAGCCTCCCCTTTTT-3’
HOXC8	5’-GGGGAGACGGAGAAACAGTG-3’	5’-GTGTGGTGAGAGACAGACCG-3’
TNFRSF21	5’-GAGAGGGAGGTTGCTGCTTT-3’	5’-CAACATCGTTTCTCCGGTGC-3’
EZH2	5’-TTGGTGGGGTCTTTATCCGC-3’	5’-GGCGCACTTCCTCCTGAAT-3’
UTX	5’-CGTGTCGTATCAGCAGGAAA-3’	5’-CACCCCAGTAACCTTCAGGA-3’
JMJD3	5’-GGTTCCAATGAGACAGGGCA-3’	5’-GATAAGAGTGCCCGCTACCC-3’

Supplementary Figure 1. CDKI-73 increases H3K27me3 through CDK9 inhibition. (A) Methylated histone level in Karpas-422, MCF-7, SGC-7901, MDA-MB-453, and SW620 cells after exposure to CDKI-73/Flavopiridol for 24 h. (B) The influence of CDK9 knock down on the protein level of H3K27me3 in Pfeiffer. (C, D) The H3K27ac level in Karpas-422 cells after CDKI-73 treatment for 24 h and CDK9 knock down.

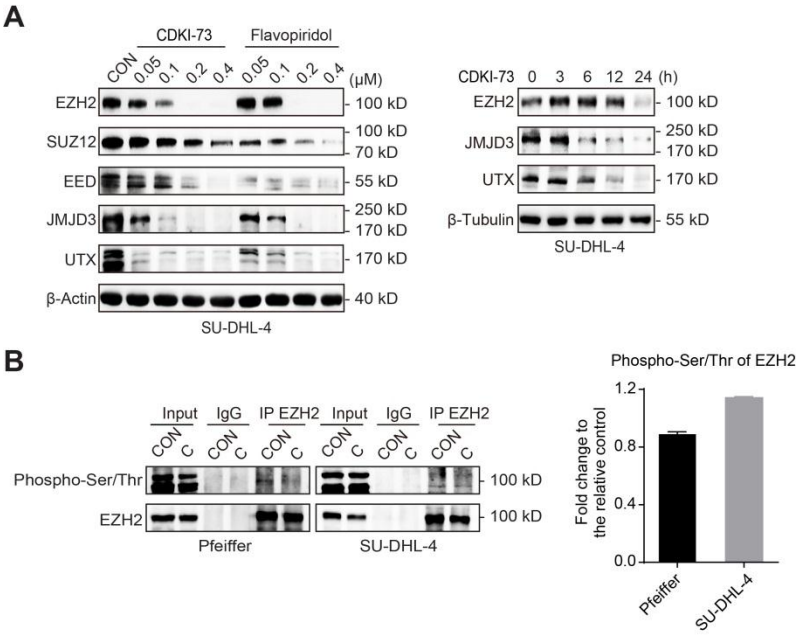
Supplementary Figure 2. Transcriptional repression contributed to H3K27me3 elevation via CDK9 inhibition. (A) Protein levels of H3K27-related methyltransferases and demethylases in SU-DHL-4 cells after exploring to CDKI-73 or Flavopiridol. (B) Quantitative assessment of immunoprecipitation (IP) for Phosphor-Ser/Thr of EZH2 in SU-DHL-4 when pretreated with 0.1 μM CDKI-73 for 12 h. EZH2 was used as a loading control. C: CDKI-73. All data are representative of at least three independent experiments.

Supplementary Figure 3. CDK9 inhibition synergies with EZH2 inhibition. (A) Impact of CDK9 knock down on the sensitivity of Pfeiffer cells to EPZ6438. Western blotting analysis of CDK9 depletion. (B) Average CI values of Pfeiffer, Karpass-422, SGC-7901, MCF-7 and SW620 cell lines for combination of different CDK9 inhibitors and EPZ6438/GSK126. Cells were treated as Figure 4D mentioned. (C) Impact of CDK9 knock down on the proliferation of EZH2 low expressed MCF-7 cells. Western blotting analysis of CDK9 and EZH2 depletion. All data are representative of at least three independent experiments.

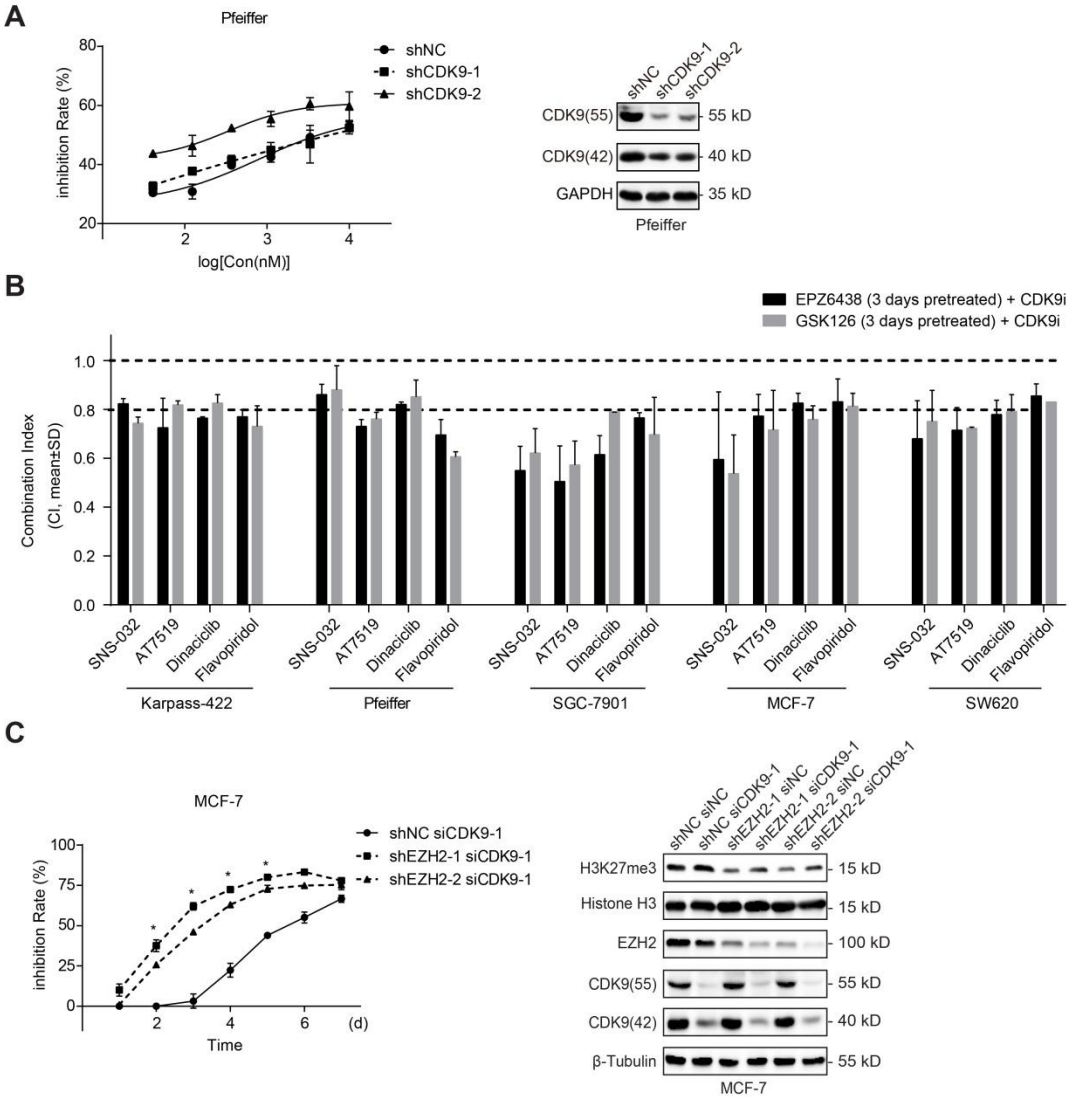
Supplementary figure 1



Supplementary figure 2



Supplementary figure 3



Supplementary Table S1. Nomalized CDKI-73/DMSO ratios in Mass Spectrometry

Sites	Ratio	Sites	Ratio	Sites	Ratio
H3K9ac	0.73	H3K36me1	0.90	H2AK5ac	0.61
H3K9me3	1.03	H3K56ac	1.07	H2AK7ac	1.06
H3K9me2	0.67	H3K79ac	0.97	H2AK9ac	0.61
H3K9me1	0.99	H3K79me2	0.98	H2AK11ac	1.02
H3K14ac	0.70	H3K79me1	1.11	H4K5ac	0.89
H3K18ac	1.05	H2BK16ac	0.64	H4K8ac	0.83
H3K18me1	1.36	H2BK20ac	0.85	H4K12ac	0.73
H3K23ac	1.20	H2BK23ac	0.65	H4K16ac	0.97
H3K27ac	0.42	H2BK34ac	1.10	H4K20me2	0.90
H3K27me3	1.29	H2BK116ac	1.13	H4K20me	0.72
H3K27me2	0.67	H2BK120ac	1.16	H4K31ac	0.94
H3K27me1	0.68	H2AK4ac	1.07	H4K77ac	1.00

Supplementary Table S2. Plasmids in stable transfection

Gene	Plasmid number
CDK9-1	TRCN0000000494
CDK9-2	TRCN0000000495

All the plasmids were purchased from Sigma-Aldrich

Supplementary Table S3. siRNA sequences in RNA interference

Gene	siRNA sequence (sense)
CDK9-1	5’-UAGGGACAUGAAGGCUGCUAA-3’
CDK9-8	5’-GGGCAUUUGAGUUUAUAUC-3’
CDK9-103	5’-GGUGCUGAUGGAAAACGAGTT-3’
CDK1	5’-CGGGAAAUUUCUCUAUUAA-3’
CDK2	5’-GGAGCUUGUUAUCGCAAAU-3’
CDK4-1	5’-GGGCAAUCUUUGCCUUUAU-3’
CDK4-2	5’-CCAGGACCUAAGGACAUAU-3’
CDK7-1	5’-GCCUACAUGUUGAUGACUC-3’
CDK7-2	5’-GAGUUUCCCUGGAAUACCU-3’