Extended exposure to low doses of azacitidine induces differentiation of leukemic stem cells through activation of myeloperoxidase

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Received: May 2, 2023.
Accepted: October 30, 2023.
Early view: November 9, 2023.

https://doi.org/10.3324/haematol.2023.283437

Supplemental figure legends

Figure S1. Extended-dose Aza leads to a more sustained hypomethylation. (A) OCI-AML2, MV-4-11, and SKM-1 cells treated with extended (blue), or conventional (green) Aza regimen for 5 days were subjected to whole genome bisulfite sequencing to assess genome wide methylation changes. (B) Schematic of dosing mice used for [^{14}C] Aza incorporation. (C and D) Measurements of Aza incorporation using the conventional or extended dose as indicated. The study samples were analyzed for radioactivity in DNA. Results were corrected for background values (5 μl water + 20μl 1.97 mg/ml ^{14}C-paracetamol) and then converted to pgEq Aza using the specific activity of the dose formulation (37 MBq/g) and to pgEQ/μg DNA using the absolute yield of DNA.

Figure S2. Bioluminescence imaging results at (A) Day 15, 18 and 21 (B) post injection are presented as group mean ± SEM (n = 10/group). Percentage of C1498-GFP+ cells on Day 25 in the blood.

Figure S3. Aza treatment induces differentiation in LSCs. (A) Bar graph of flow cytometry data showing CD34+38- and CD34+38+ cell populations (% of cells) from 3 independent experiments using OCI-AML20 cells with the Aza dosing as indicated. Statistical analysis was performed using two-way ANOVA (B) Flow cytometry assessment, using Annexin V and live/dead stains, was done to assess cell death in OCI-AML20 following Aza treatment, day 7. (C) Primary AML sample harboring TP53 and TET2 mutations were treated with the conventional or extended dose. On day 7, flow cytometry was performed to identify CD34 and CD38 fractions.

Figure S4. LogFc of mRNA of secreted factors from LSCs. (A) Bar graph depicting logfc of genes from RNAseq data whose protein secretion changed by more than 20% following Aza treatment, relative to control on days 5 and 7. (B) Bar graphs representing the percentage of progenitor population in OCI-AML20 treated with the indicated Aza dosing with MPO-IN-28 or (C) LSCs treated with NAC on day 7 from three independent experiments. Statistical analysis was performed using two-way ANOVA. * Indicates an adjusted p value of < 0.01

Supplemental methods

Capillary Electrophoresis

Briefly, approximately 5 million cells were lysed with 300–500 µL of cold radioimmunoprecipitation assay (RIPA) buffer containing a protease and phosphatase inhibitor cocktail and vortexed. Samples were then centrifuged at maximum speed at 4˚C for 30 minutes. The supernatant was collected, and protein concentration assessed by bicinchoninic acid (BCA) assay. For capillary electrophoresis, 4 µL of 1–1.5 mg/mL of sample was ran in Wess or Jess, according to manufacturer's protocol, using antibodies to assess specific proteins.

Single Cell RNA-seq

Single-cell suspensions were converted to barcoded single-cell RNA (scRNA)-seq libraries by using the Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index), Gel Bead & Multiplex Kit, and Chip Kit (10x Genomics; Pleasanton, CA), loading an estimated 15,000 cells per library and following the manufacturer's instructions. The final libraries were profiled using the Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA) and sequenced using Nova S4 (Illumina; San Diego, CA) to obtain 10 bp dual index, approximately 200 million reads per library. scRNA-seq data for each sample were processed with CellRanger count (CellRanger 2.1.0; 10x Genomics) using a custom reference package based on mouse and human reference genome, subtracting mouse transcript reads. Subsequent data analysis was carried out in R 3.5.1 and the Seurat package (v2.3.4).

RNA-seq

Control and drug treated cells were lysed by the addition of 350 µL of β-ME containing buffer RLT. Samples were then snap frozen before processing and analysis. Sequencing libraries were created using the IlluminaTruSeq Stranded messenger RNA (mRNA) method, which preferentially selects for messenger RNA by taking advantage of the polyadenylated tail. Libraries were sequenced using the Illumina sequencing-by-synthesis platform, with a sequencing protocol of 100 base-pair (bp) paired-end sequencing and total read depth of 30M reads per sample.

Whole Genome Bisulfite Sequencing

Control or Aza treated cells were pelleted and snap frozen before analysis. DNA extraction from cells was performed with QIAmp DNA kit (Qiagen; Hilden, Germany) and quantified with Qubit 2.0 DNA HS Assay (ThermoFisher; Waltham, MA) followed by quality assessment using Fragment analyzer genomic DNA Assay (Agilent Technologies, Inc.; Santa Clara, CA). Genomic

DNA was bisulfite converted with EZ DNA Methylation-Gold Kit (Zymo Research; Irvine, CA). Library preparation was then performed using Accel-NGS® Methyl-Seq DNA Library kit (Swift Biosciences; Ann Arbor, MI) and quality assessed by TapeStation HSD1000 ScreenTape (Agilent Technologies Inc.). Equimolar pooling of libraries was performed based on QC values. Samples sequencing was done on an Illumina® NovaSeq S4 with a read length configuration of 150PE for 1B PE reads per sample (500M in each direction). A 20% PhiX Spike In was added during the sequencing to ensure sequencing quality.

Bioinformatic analysis of Whole-Genome Bisulfite Sequencing (WGBS) and RNA-seq

Bismark methylation counts were restricted to those CpG islands showing at least one methylated residue and at least one unmethylated residue across all samples, and whose total depth was at least seven reads within each sample. Beta values were computed as the methylated residue fraction.

Differences in coverage were assessed by taking the maximum absolute difference between treatment and matched DMSO control and performing 1,000 permutations. The resulting distribution of maximum difference statistics was closely approximated by a gamma distribution; and final p-values were estimated by fitting the parameters of a gamma distribution to the permutated values (per CpG) and computing the distribution survival value of the observed statistic. The resulting distribution of p-values was deflated, and the top hits were on un-localized contigs. We therefore did not control for coverage in downstream analyses.

Regional methylation values for promoters, enhancers, and gene bodies were computed as the total number of methylated reads within an interval, divided by the total number reads. Gene bodies were defined by RefSeq MANE select genes, and promoter regions were defined as 1,000bp upstream of the first base of the gene definition. Enhancer regions were defined by the AML track of EnhancerAtlas (1).

Sample outliers were assessed by taking the principal components of the top 500 most variable CpG sites (by coefficient of variation) and computing the square of the loadings across the top two principal components. Those samples with a final chi-square of 8 or higher were considered as putative outliers and confirmed visually on the loading plots. This process was repeated until no flagged or visually confirmed outliers are present. This process resulted in the exclusion of one sample.

Differential expression was performed using LIMMA with the VOOM transform method, separately within each cell line and treatment date (2). Only the top 12,000 genes (by median expression) were considered for this test. Differential methylation utilized the computed beta values in a linear

model, separately within each cell line and date. TREAT is applied with a log fold change threshold of 1.5 (gene expression) or a beta change of 0.25 (methylation). Gene set over-representation analyses and gene set enrichment analyses were performed using the R package piano using the Stouffer statistic for gene set enrichment analysis (GSEA). Transcription factor enrichment analysis (TFEA) was performed using the TFEA python package.

Comparisons between days were performed directly via LIMMA (expression) or a linear model (methylation) using the contrast day 3 to day 5, and comparisons between treatments at a particular timepoint used to contrast conventional versus extended-dose Aza.

In Vivo Bioluminescence Imaging

Luciferase activity was measured in animals using an IVIS® SpectrumCT (PerkinElmer, Inc., Waltham, MA). On the day of imaging, animals were injected VivoGlo™ D-Luciferin substrate (Promega Corporations, Madison, WI; Catalog P1043). Sedated animals were used for ventral image acquisition 10 minutes after receiving luciferin substrate injections. Acquired image was analyzed and exported using Living Image software 4.5.1. (PerkinElmer, Inc.). Flux equaling the radiance (photons/s [p/s]) in each pixel summed or integrated over the region of interest area (cm2) x 4π was used to represent tumor burden; the region of interest included the entire animal.

References:

1. Gao T, He B, Liu S et al. EnhancerAtlas: a resource for enhancer annotation and analysis in 105 human cell/tissue types. Bioinformatics. 2016;32(23):3543-51.

2. Ritchie ME, Phipson B, Wu D et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43(7):e47.

Figure S1: Extended -dose Aza leads to a more sustained hypomethylation

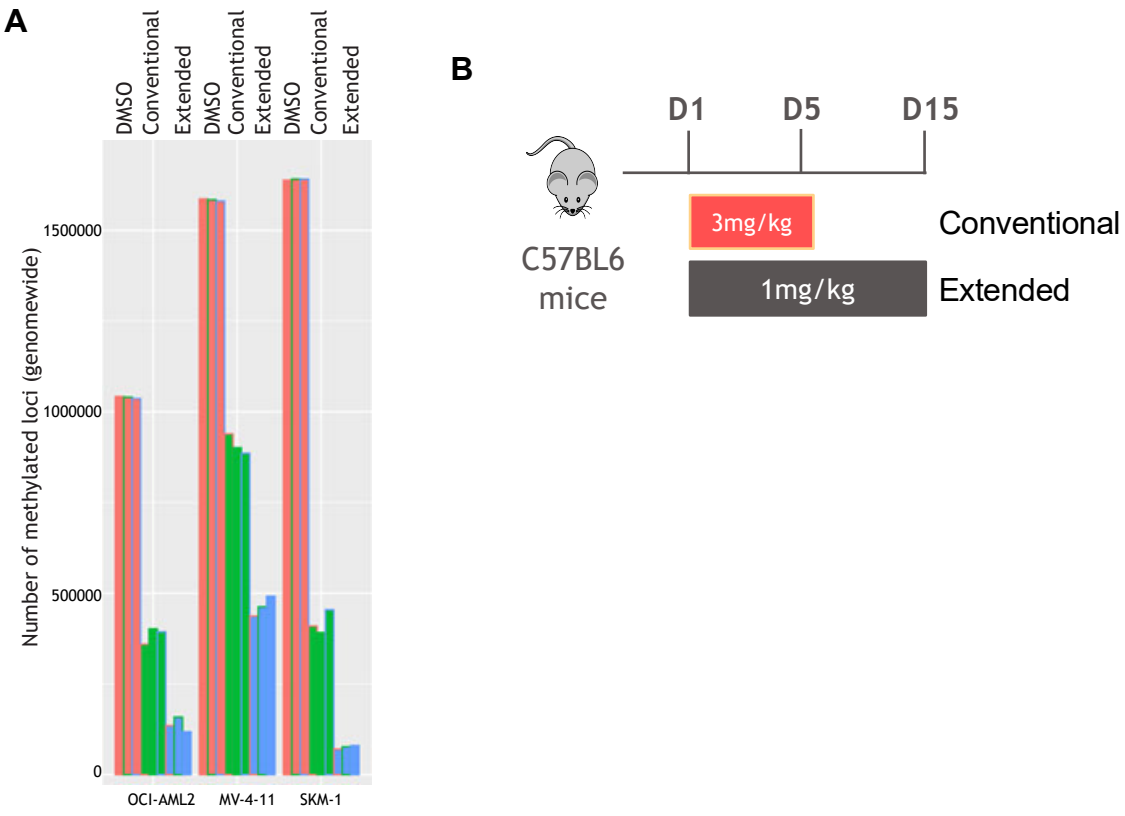
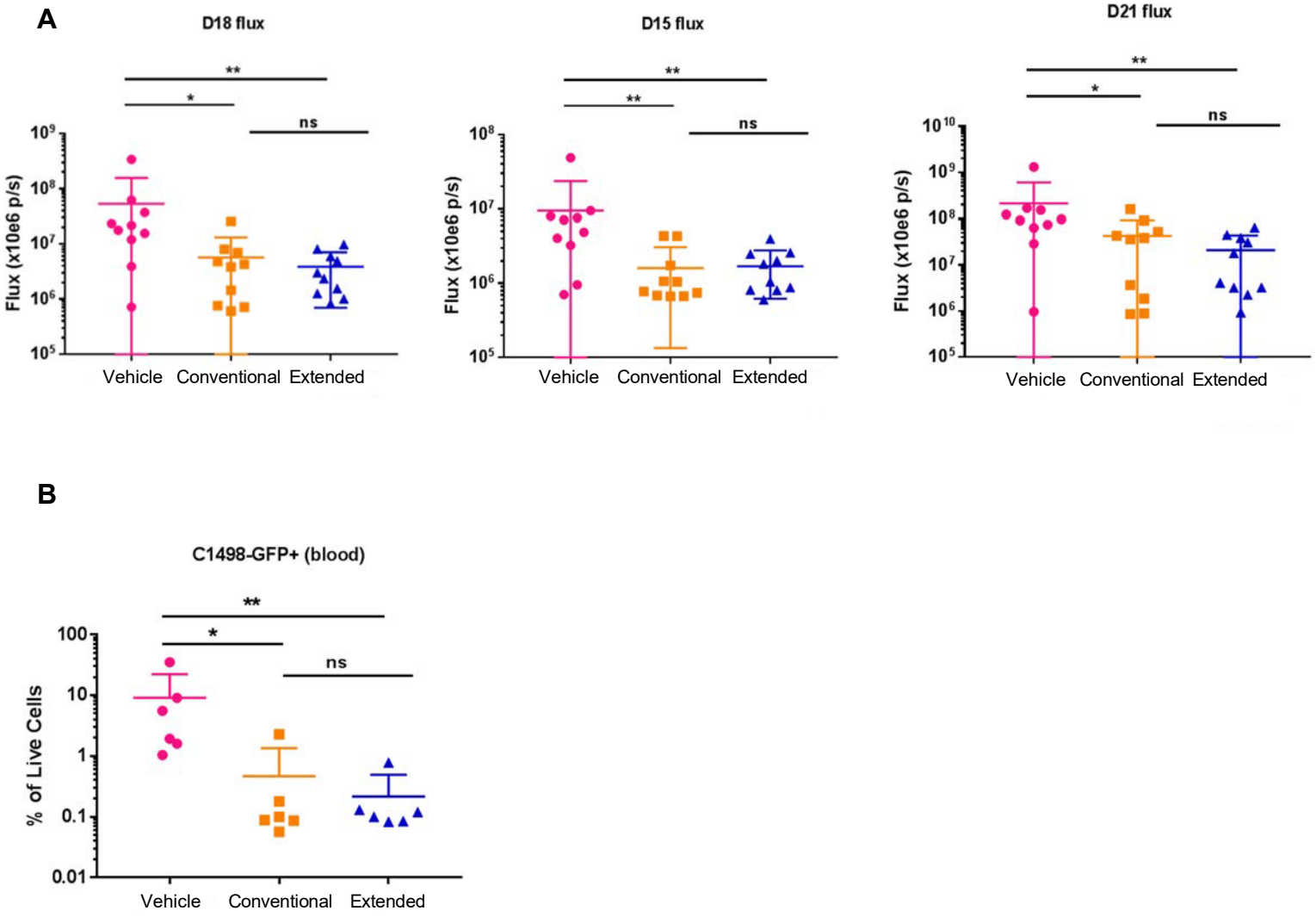
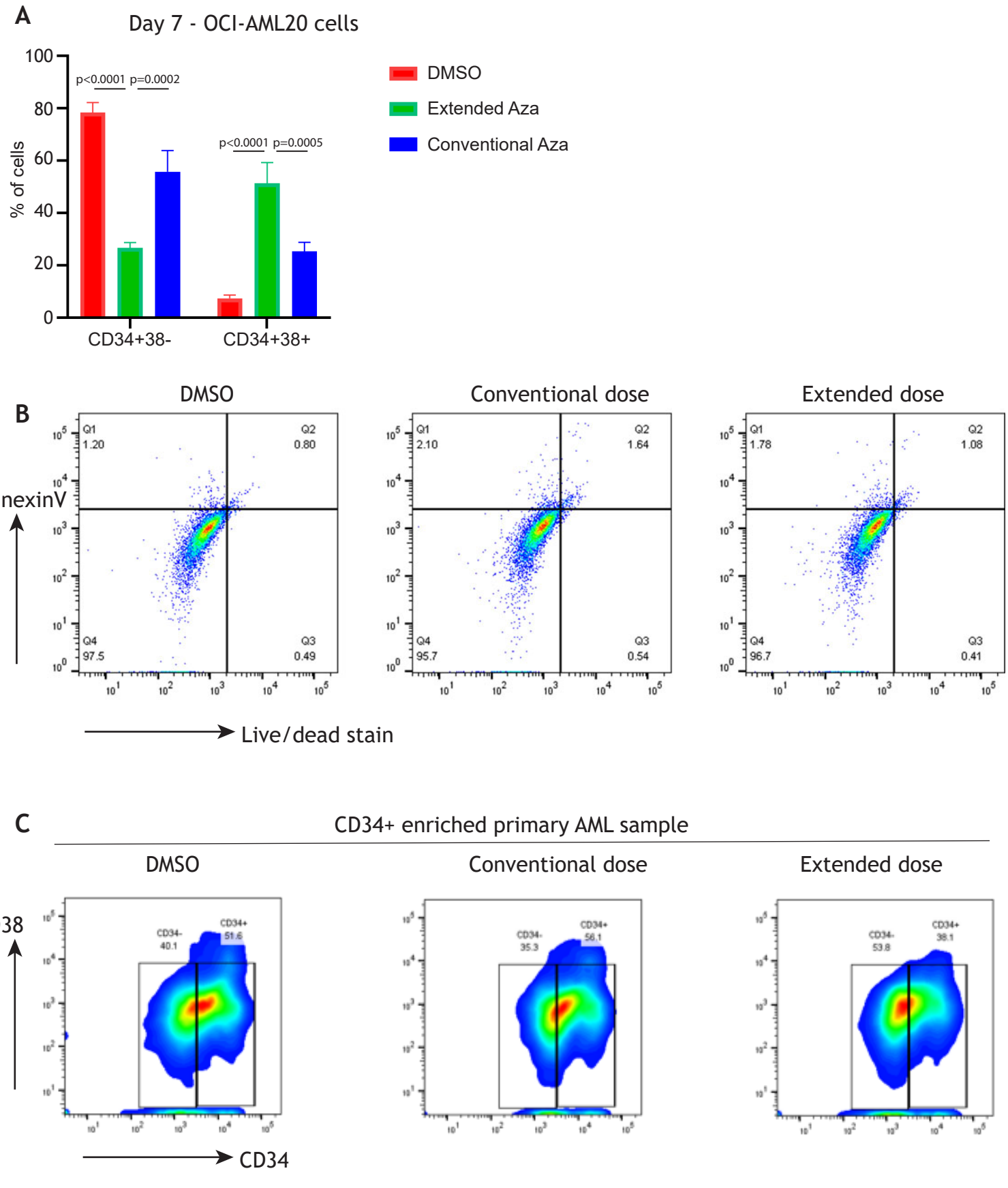


Figure S2: Extended -dose Aza has similar anti-leukemic effect as convetional-Aza

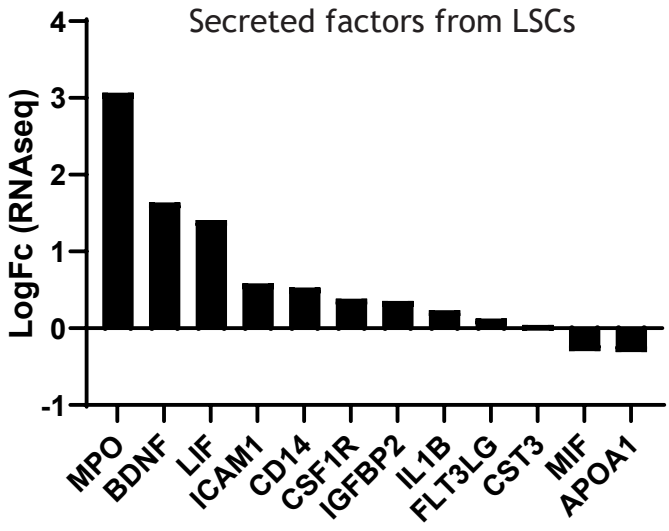


Supplemental Figure S3: Extended-dose Aza treatment induces differentiation in LSCs



Supplemental Figure S4: MPO is upregulated in LSCs treated with extended dose Aza

A



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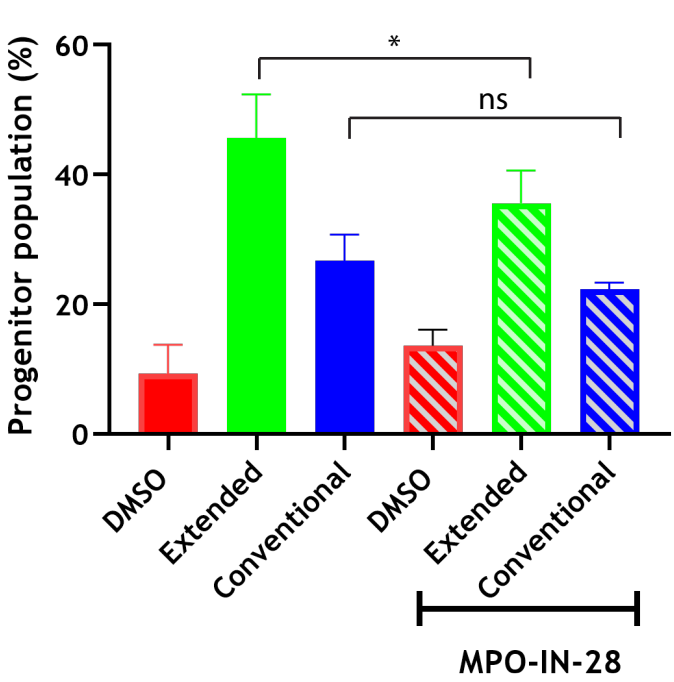
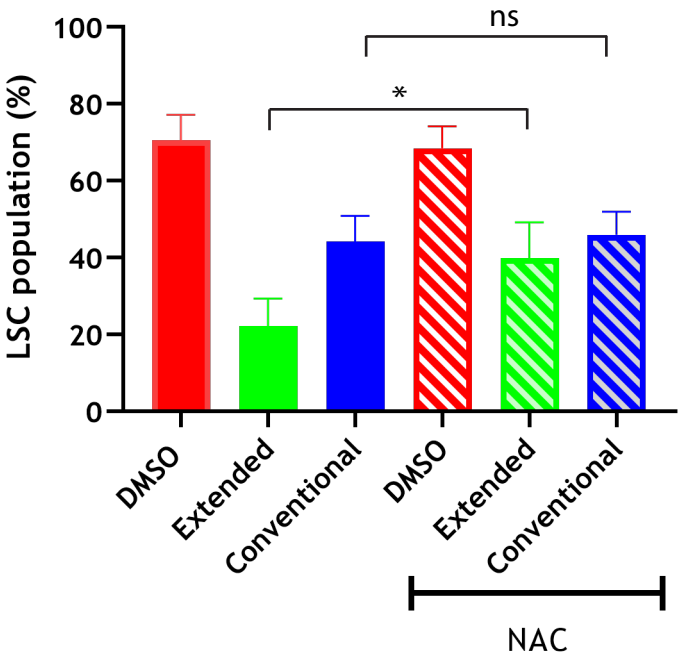


C



Supplementary table 1: Comparison of beta values of methylation across cell lines and treatments

Cell Line	Day	Comparison	Median of Betas for all CpG sites	Ratio of number of CpG sites with Beta> 0.3	Ratio of number of CpG sites with Beta> 0.5	Ratio of number of CpG sites with Beta> 0.7	
OCI-AML2	3	DMSO vs Conventional	1.86667	1.22491	1.78185	3.77392	
OCI-AML2	3	DMSO vs Extended	1.33333	1.03475	1.20786	1.73205	
OCI-AML2	3	Conventional vs extended	0.71429	0.84476	0.67787	0.45895	Conventional Aza demethylates DNA more potently than extended Aza at day 3 in OCI-AML2 and MV411. Extended Aza demethylates DNA more potently than conventional Aza at day 3 in SKM1.
MV-4-11	3	DMSO vs Conventional	1.42657	1.05208	1.19593	1.74568	
MV-4-11	3	DMSO vs Extended	1.38462	1.05640	1.18287	1.59548	
MV-4-11	3	Conventional vs extended	0.97059	1.00411	0.98908	0.91396	
SKM1	3	DMSO vs Conventional	1.74107	1.11483	1.57543	3.08587	
SKM1	3	DMSO vs Extended	1.87500	1.12538	1.67354	4.18928	
SKM1	3	Conventional vs extended	1.07692	1.00946	1.06228	1.35757	
OCI-AML2	5	DMSO vs Conventional	1.50000	1.12047	1.33300	2.00050	
OCI-AML2	5	DMSO vs Extended	2.33333	1.39475	2.20448	4.45872	
OCI-AML2	5	Conventional vs extended	1.55556	1.24479	1.65378	2.22880	
MV-4-11	5	DMSO vs Conventional	1.34815	1.04654	1.14366	1.50420	Extended Aza demethylates DNA more potently than conventional Aza at day 5 in all cell lines
MV-4-11	5	DMSO vs Extended	1.73333	1.16289	1.53101	2.51882	
MV-4-11	5	Conventional vs extended	1.28571	1.11117	1.33869	1.67452	
SKM1	5	DMSO vs Conventional	1.52778	1.06702	1.32358	2.40849	
SKM1	5	DMSO vs Extended	2.20000	1.27271	2.54993	8.51010	
SKM1	5	Conventional vs extended	1.44000	1.19277	1.92654	3.53337	
						Comparisons of conventional vs extended highlighted	

Values <1 represent lower methylation in the first term of column "Comparison" (i.e., for DMSO vs conventional, DMSO is first term and Conventional second term)

Values >1 represent higher methylation in the first term of column "Comparison" (i.e., for DMSO vs Conventional, DMSO is first term and Conventional second term)