SUPPLEMENTARY APPENDIX

The thymidine dideoxynucleoside analog, alovudine, inhibits the mitochondrial DNA polymerase γ, impairs oxidative phosphorylation and promotes monocytic differentiation in acute myeloid leukemia

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Running Title

POLG inhibition promotes AML differentiation

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KEYWORDS

Alovudine; POLG; nucleoside analog; AML; differentiation

Supplemental methods

Measurement of mitochondrial and nuclear DNA

To measure levels of mitochondrial DNA (mtDNA), genomic DNA was isolated from the various cell lines using the DNAeasy Blood and Tissue kit (Qiagen, Germantown, MD). The relative mtDNA levels were determined by quantitative real-time polymerase chain reaction (qPCR) using the mtDNA-encoded human NADH dehydrogenase 1 (ND1) gene normalized to the nuclear-encoded human beta globulin gene (HGB). Primer sequences for mt-ND1-F: 5'-AACATACCCATGGCCAACCT-3' and ND1-R: 5'-AGCGAAGGGTTGTAGTAGCCC-3', and for HGB-F: 5'-GAAGAGCCAAGGACAGGTAC-3' and HGB-R: 5'-CAACTTCATCCACGTTC ACC-3'. The reactions were performed on an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA).

Metabolic analysis

Basal oxygen consumption rate (OCR) and extracellular acidification rate were measured using Seahorse Bioscience XF96 Extracellular Flux Analyzer according to the manufacturer's protocol (Agilent, Santa Clara, CA). XF96e Seahorse Biosciences plates pre-coated with Cell-Tak BD (at 0.16 μg/well) were used according to the manufacturer's instructions (BD Biosciences, Mississauga, CA). Cells were resuspended in unbuffered Alpha-MEM media supplemented with 2% FBS and plated at 1.9×10^5 cells/well on the day of the assay. The results represent the average basal OCR (pMol/min) calculated from the first three measurements taken at 3 minutes intervals.

Tissue Histology

SCID mice with OCI-AML2 xenografts were treated with alovudine in fixed dose as described previously. Following treatment, mice were sacrificed and organs were collected, fixed in 10% formalin, paraffin embedded, sectioned and stained with hematoxylin and eosin. The stained samples were scanned using Aperio Scanscope X and analyzed using Aperio ImageScope (Concord, ON) at 20× magnification.

ATP quantification

Relative ATP levels in cells were determined using the CellTitre-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), according to the manufacturer instructions.

DNA Methylation

DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD).

1µg of DNA was spotted in duplicate onto 0.2 µm nitrocellulose membrane (GE, Chicago, IL) and crosslinked using an FB-UVXL-1000 UV Crosslinker (Fisher, Markham, ON) for 20 minutes. Following blocking in 5% milk-TBST for 1 hour at room temperature, membranes were incubated with anti-5-Hydroxymethylcytoseine (1:250) (Active Motif, Carlsbad, CA) and anti-dsDNA (1:10,000) (Abcam, Cambridge, MA) on a rocker overnight at 4ºC. Following one hour incubation with secondary anti-rabbit (1:2000) or anti-mouse (1:10,000) antibodies [GE Healthcare (Buckinghamshire, UK], proteins were detected by chemiluminescence.

Immunoblotting

Cells were lysed in lysis buffer containing 1.5% (w/v) n-dodecyl-β-maltoside (n-DBM) (Sigma Aldrich, St. Louis, MO) with protease inhibitor mixture (Roche, Mississauga, CA). Protein was quantified with DC protein assay (Bio-Rad, Mississauga, ON, Canada). For the detection of COX I, II, IV proteins, the lysates were subjected to electrophoresis on SDS-PAGE and transferred with (3-[Cyclohexylamino]-1-propanesulfonic acid) CAPS buffer (pH 10.5) to a PVDF Membrane at 70 V for 1 hour. Following the transfer, blots were blocked for 1 hour with 5% milk in PBST at room temperature, followed by overnight incubation at 4°C with primary antibodies. Blots were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK) for 1 hour at room temperature. Protein bands were visualized by chemiluminescence.

The following antibodies were used: mt-COX I (SC 58347) – Santa Cruz Biotechnology Inc. (Santa Cruz, California), mt-COX II (Ab110258) – Abcam (Cambridge,

Massachusetts), mt-COX IV (A21347) – Life Technology (Burlington, Canada), β-tubulin (SC9104) – Santa Cruz Biotechnology Inc. (Santa Cruz, California).

POLG knockdown with shRNA

Bacterial stocks containing pLKO.1 vectors with shRNA against POLG gene were purchased from Sigma Aldrich (Oakville, Ontario). Genetic knockdown of POLG in OCI-AML2 cells was achieved using lentiviral transduction of short hairpin RNAs (shRNA) delivered by pLKO.1 vector with a puromycin resistance marker. Lentiviral transduction was performed as previously described[1]. Briefly, OCI-AML2 cells (5 x 10^6) were centrifuged and re-suspended in 5 mL of medium containing protamine sulfate (5 µg/mL) and 2 mL of virus for an overnight incubation (37 C, 5% CO2). The following day, cells were centrifuged, washed and incubated in media with puromycin (1µg/mL) for 72 hours to select transduced cells.

Cell proliferation assay

Proliferation assays were performed by seeding 100,000 cells/ml for cell lines, and 600,000 cells/ml for patient samples and PBSCs. Alovudine was supplied by Medivir AB (Huddinge, Sweden) and dissolved in DMSO. Alovudine was added to fresh culture medium twice weekly when cells were passaged, for a total period of 10 days for cell lines, and 6 days for primary AML cells. On days 3, 6 and 10 (cell lines) viable AML cells were counted by trypan blue exclusion staining and the growth and viability of normal hematopoietic cells were determined by CellTiter-Fluor (Promega Corporation, Madison, WI) as per the manufacturer's instructions.

Flow cytometry

Cell death was quantified by Annexin V-fluoroscein isothiocyanate (FITC) and Propidium iodide (PI)-staining (Biovision Research Products, Mountain View, CA) according to manufacturer's instructions.

For differentiation assessment cells were stained with anti-CD11b-APC 7AAD to exclude dead cells; all from BD Biosciences (Becton Dickinson, San Jose California. Mitochondrial mass was assessed by Mitotracker Deep Red FM staining (Invitrogen, Burlington, Canada). Cells were stained using 100nm dye for 30 minutes at 37 degrees,

pelleted and re-suspended with Annexin V-FITC to exclude non-viable cells from the analysis. Mitochondrial mass was quantified as mean fluorescence intensity of the viable (Annexin V negative) cell population.

For cell cycle analysis $2x10^6$ cells were washed with PBS and fixed in cold 80% ethanol in PBS. After overnight incubation at -20 degrees, cells were washed in PBS and treated with 5ug/ml DNAse free RNAse A (Hoffmann-La Roche, Mississauga, ON) in PBS at 37 degrees for 30 minutes. Propidium iodine (Sigma-Aldrich, St. Louis, MO) was then added to the cells at a concentration of 5ug/ml and DNA content was analyzed by flow cytometry.

Flow cytometry analysis was performed using a Fortessa or a BD FACSCanto flow cytometer (BD Biosciences, Mississauga, ON, Canada) and FlowJo software (Tree Star, Ashland, OR, USA)

Quantitative reverse transcription polymerase chain reaction (RT_qPCR)

RNA was isolated from cell pellets using the RNEasy Plus Mini kit (Qiagen, MD, USA). First strand cDNA was synthesized with random primers using the SuperScript IV Kit (Invitrogen, Burlington, ON) as per manufacturer's instructions. RT-qPCR was conducted as previously described [1]. The cDNAs encoding human cytochrome C oxidase (COX I, COX II, COX IV), POLG and ribosomal RNA subunit 18 (18S) were amplified using the following primer pairs: COX I-F: 5'-CTATACCTATTATTCGGCGCATGA-3', COX I R: 5'-CAGCTCGGCTCGAATAAGGA-3', COX II-F: 5'-CTGAACCTACGAGTACACCG-3', COX II-R: 5'-TTAATTCTAGGACGATGGGC-3', COX IV-F:5'-GCCATGTTCTTCATCGGTTTC-3', COX IV R: 5'-GGCCGTACACATAGTGCTTCTG-3', 18s-F: 5' AGGAATTGACGGAAGGGCAC-3', 18s-R: 5'-GGACATCTAAGGGCATCACA-3', POLG-F: 5'-GGAGGAGTTCCTGCTCACTG-3', and POLG-R: 5'-GAGGCAGCTTGAAAAACCAG-3'.

Evaluation of hematopoietic progenitor cells by colony assay:

Primary AML and normal hematopoietic cells were obtained as described in the methods section. All primary cells were cultured in MyeloCult™ H5100 media (Stem Cell Technologies) supplemented with rh-IL7 (20 ng/ml), rh-IL6 (20 ng/ml), rh-FLT3-L (10 ng/ml), rh-GM-CSF (20 ng/ml), rh-SCF (100 ng/ml), rh-IL-3 (10 ng/ml), and rh-G-CSF (20 ng/ml) (R&D Systems, Minneapolis, MN and Miltenyi Biotec, Auburn, CA). Cells ($4x10^5$/ml) were pre-treated with 500 and 2000 nM of alovudine (or vehicle) for 72 hrs, washed and plated for colony assay in the presence of alovudine (or vehicle) in Methocult 04434 (StemCell Technologies). Colonies were scored as CFU-L if containing at least 10 cells while BFU-E and CFU-GM if at least 50 cells each.

Statistical analysis

All experiments were performed at least three times with technical replicates. Statistical analyses were performed using Graph Pad Prism 6.03 (La Jolla, CA, USA).

Supplemental tables

Table S1 (Related to Figure 2A-C): AML patient characteristics

Sample #	Patient ID	Age at diagnosis	Gender	Diagnosis	Cytogenetics	Molecular
1	162111	19	Male	AML with mutated NPM1	45,X,-Y[9]/46,XY[11]	NPM1 positive; FLT3-ITD negative; CBFB-MyH11 undetectable
2	100647	54	Female	AML with mutated NPM1	46,XX[10]	NPM1 positive, FLT3-TKD negative
3	110540	84	Male	AML	46,XY[20]	Not done
4	162131	64	Female	AML with myelodysplasia-related changes (previous MDS)	46,XX[20]	NPM1 negative; FLT3 ITD positive (Low)
5	090784	62	Female	AML with mutated NPM1	46,XX[20]	NPM1 positive, FLT3-IT negative
6	100770	84	Female	AML	46,XX,del(5)(q13q33)[5]/50,idem,+5,+8,+21[14]/46,XX[2]	Not done
7	090658	81	Female	AML	46,XX[14]	Not done
Xenograft	090240	54	Female	AML without maturation	52,XX,+2,+9,+10,+13,+14,+1[20]	Not done

Supplemental Figures

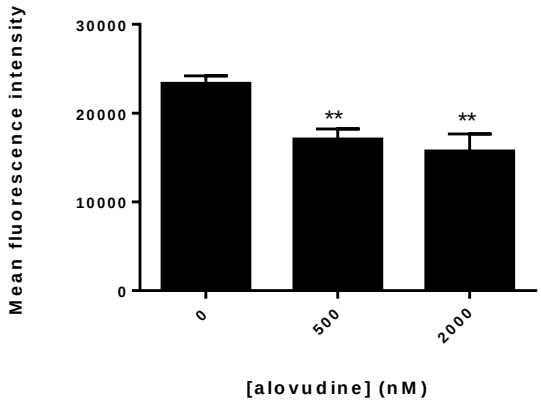


Figure S1 (Related to Figure 1): Effects of alovudine on mitochondrial mass in acute myeloid leukemia

OCI-AML2 cells were treated with increasing concentrations of alovudine for 6 days. Miotochondrial mass was assessed by Mitotracker Deep Red FM staining as described in supplementary “Methods” and analyzed by flow cytometry. Data are mean fluorescence intensity $\pm$ SD from two representative experiments

**P < 0.01 using Dunnett’s multiple comparisons after one-way ANOVA

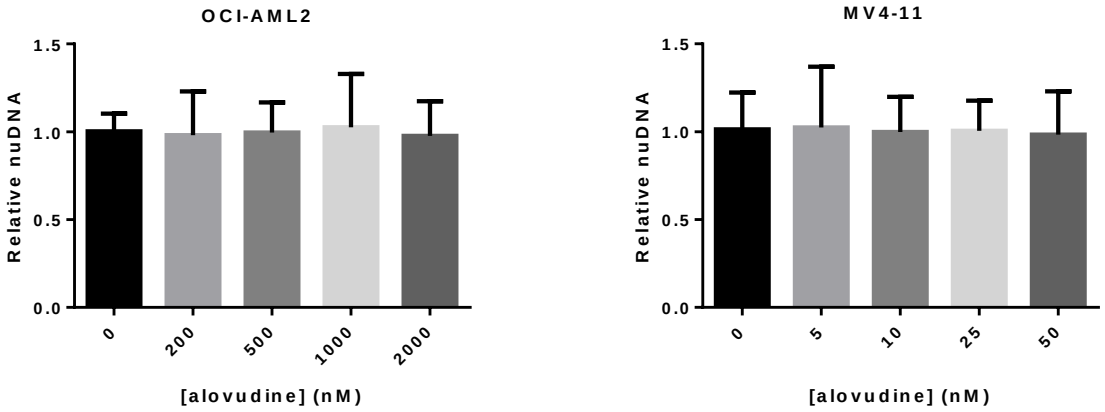


Figure S2 (Related to Figure 1): Effects of alovudine on nuclear-DNA

OCI-AML2 and MV4-11 cells were treated with increasing concentrations of alovudine for 6 days. Relative nuclear (nu)DNA content was assessed by qRT-PCR as described in supplementary "Methods". Data represent mean ± SD nuDNA relative to untreated controls from one of three representative experiments. nuDNA: nuclear DNA

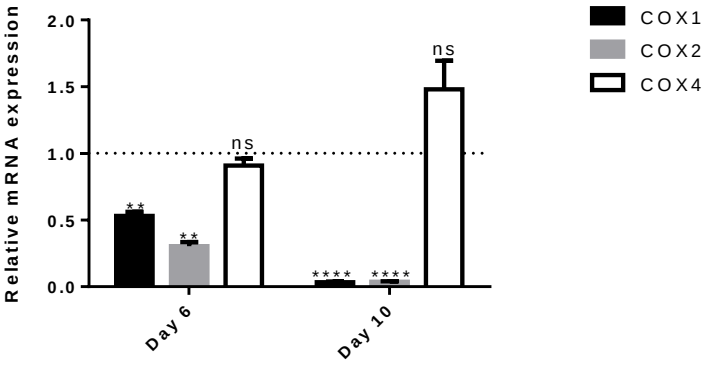


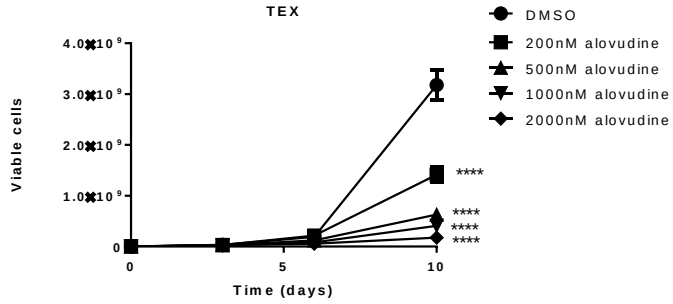
Figure S3 (Related to Figure 2): Effects of alovudine on gene expression in acute myeloid leukemia

Changes in gene expression were assessed in OCI-AML2 cells following 6 and 10 days of alovudine (2000nM) treatment, using qRT-PCR as described in in supplementary

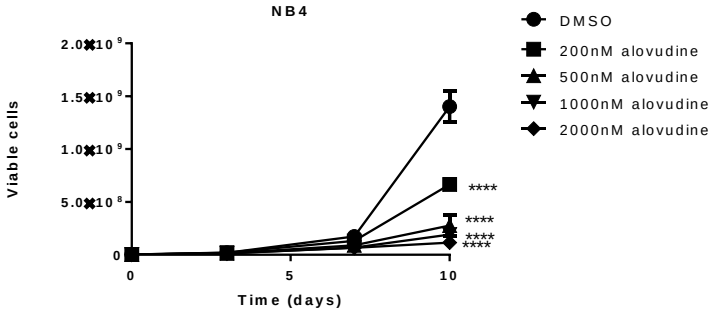
"Methods". Representative data of mRNA expression relative to untreated controls are shown as the mean ± SD

**P < 0.01 and ****P < 0.0001 using Dunnett's multiple comparisons after one-way ANOVA

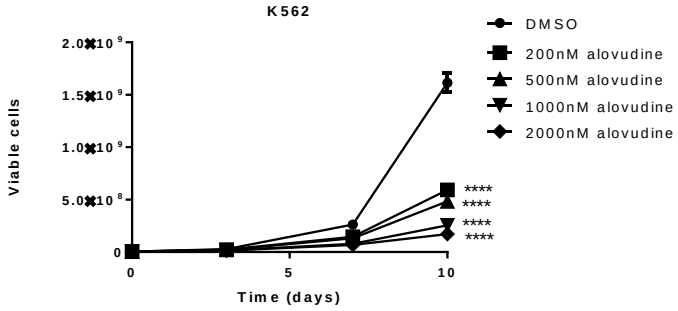
A)


TEX
Viable cells
4.0x10^9
3.0x10^9
2.0x10^9
1.0x10^9
0
0
5
10
Time (days)
DMSO
200nM alovudine
500nM alovudine
1000nM alovudine
2000nM alovudine
****
****
****
****

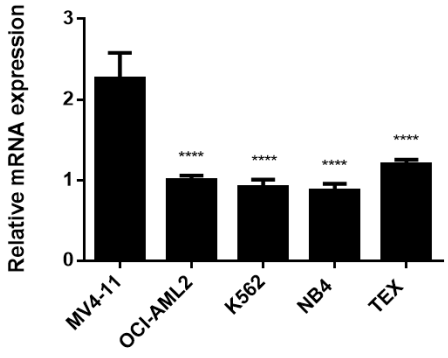
B)


NB4
Viable cells
2.0x10^9
1.5x10^9
1.0x10^9
5.0x10^8
0
0
5
10
Time (days)
DMSO
200nM alovudine
500nM alovudine
1000nM alovudine
2000nM alovudine
****
****
****
****

C)


K562
Viable cells
2.0x10^9
1.5x10^9
1.0x10^9
5.0x10^8
0
0
5
10
Time (days)
DMSO
200nM alovudine
500nM alovudine
1000nM alovudine
2000nM alovudine
****
****
****
****

D)


Relative mRNA expression
3
2
1
0
****
****
****
****
MV4-11
OCI-AML2
K562
NB4
TEX

E)

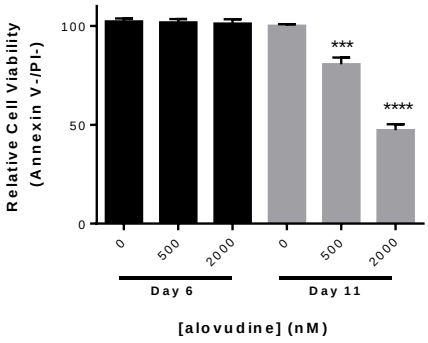

Relative Cell Viability
(Annexin V-/PI-)
100
50
0
***
****
0
500
2000
0
500
2000
Day 6
Day 11
[alovudine] (nM)

Figure S4 (Related to Figure 1F): Alovudine inhibits cell growth and viability in acute myeloid leukemia cell lines

A-C) TEX, NB4 and K562 cells were treated with increasing concentrations of alovudine for 10 days. Cell growth and viability were assessed by trypan blue exclusion staining at increasing times after incubation. Data are the mean ± SD from representative experiments

****P < 0.0001 using two-way ANOVA.

D) POLG mRNA expression was assessed by RT-qPCR in MV4-11, OCI-AML2, K562, NB4 and TEX cells. Data represent the average mean ± SD POLG expression relative to OCI-AML2 cells

E) OCI-AML2 cells were treated with alovudine for 11 days. Annexin V staining was assessed by flow cytometry. Data represent the average mean ± SEM.

***P < 0.001, ****P < 0.0001 using Dunnett's multiple comparisons after one-way ANOVA.

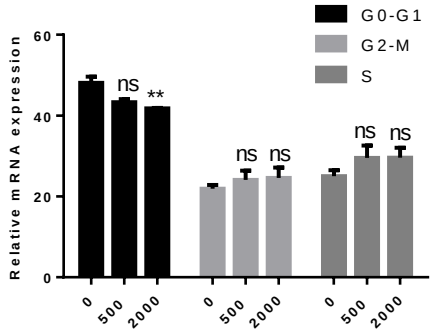


Figure S5 (Related to Figure 1): Effects of alovudine on cell cycle

OCI-AML2 cells were treated with alovudine for 6 days. Cell cycle analysis was performed as described in supplementary "Methods". Data are the mean ± SD of representative experiments.

**P < 0.01 using Sidak's multiple comparisons test after 2-way ANOVA.

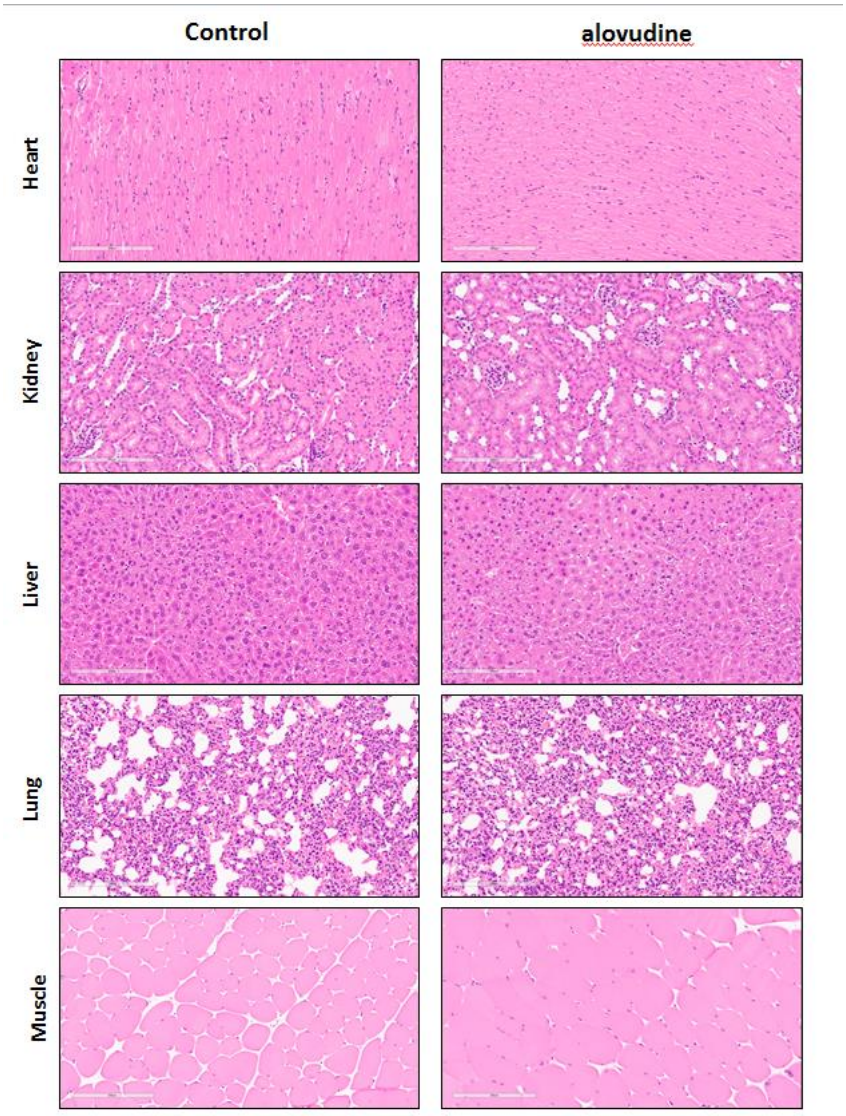


Figure S6 (Related to Figure 3): Alovudine displays efficacy in mouse models of human acute myeloid leukemia

SCID mice were treated with alovudine (50 mg/kg bid PO) or vehicle control for 14 days. Representative sections of organ histology (Heart, kidney, liver, lung and muscle) following treatment are shown, stained with hematoxylin and eosin. Scale bar=200 µm.

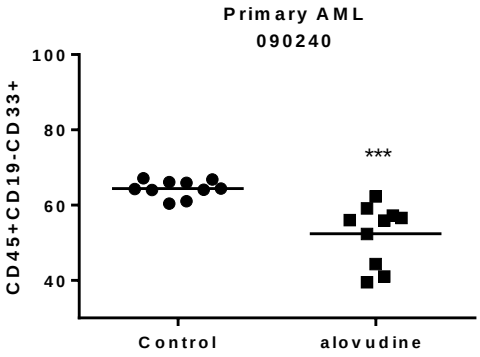


Figure S7: Effects of alovudine in a mouse model of primary human acute myeloid leukemia

Primary acute myeloid leukemia cells were injected intrafemorally into sublethally irradiated female NOD/SCID mice. Mice were treated with 25mg/kg per day of oral alovudine or vehicle control for 5 of 7 days for 24 days, a total number of 17 doses (n=10 per group). Following treatment, human leukemia cell engraftment in the left femur was assessed by flow cytometry of human $CD45^+$ $CD33^+$ $CD19^-$ cells. Line represents mean engraftment of human cells.

***P<0.001 using T-test

REFERENCES

1. Lang BF, Gray MW, Burger G. Mitochondrial genome evolution and the origin of eukaryotes. Annu Rev Genet 1999;33(1):351–397.