Supplemental Materials of *ASLAN003 for differentiation of AML*

Supplemental Methods

1. Cell lines and primary bone marrow cells

Human AML cell lines, THP-1 and KG-1, were purchased from ATCC (Manassas, VA) and MOLM-14 cell line was obtained in house. AML cell lines were grown in RPMI1640 (Biowest, France) supplemented with 10% fetal bovine serum (FBS, Biowest) in a humid incubator with 5% CO_2 at 37ºC. Bone marrow (BM) cells from newly diagnosed AML or MDS patients or relapsed AML patients were obtained at National University Hospital (NUH) in Singapore with informed consent. Normal CD34+CD38+ myeloid progenitor bone marrow cells were isolated from healthy donors at NUH by CD34 and CD38 MicroBead Kit (Miltenyi biotec, Germany). Primary BM cells were cultivated in a medium containing 80% RPMI1640 (Biowest) supplemented with 20% FBS (Biowest) and 10 ng/ml cytokines including FLT-3 ligand, IL-3, CSF, TPO and 20 ng/ml of GM-CSF at density of 5×10^5 cells/ml in a humid incubator with 5% CO_2 at 37°C. Human cytokines were purchased from Peprotech (Rocky Hill, NJ).

This study was approved by the Institutional Review Board (IRB) of National University of Singapore.

2. Drugs and chemicals

ASLAN003 was obtained in house (ASLAN Pharmaceuticals, Singapore). Brequinar (SML0113) and uridine (U6381) were purchased from Sigma-Aldrich (St. Louis, MO). ASLAN003 and brequinar were reconstituted in dimethyl sulfoxide (DMSO, Sigma) to a stock concentration of 10 mM and stored at -20°C for *in vitro* assays. Uridine was dissolved in DMSO to make 0.1 M stock. For *in vivo* study, ASLAN003 was dissolved in a vehicle consisting of PEG300 (3 parts) + 30% Captisol (7 parts). ASLAN003 was freshly prepared and used within 15 to 30 minutes after

solubilization. AP-1 inhibitor T-5224 (HY-12270) was obtained from MedChem Express (Monmouth Junction, NJ) and dissolved in DMSO.

3. FACS analysis of myeloid cell surface antigens

Cells were pretreated with increasing dose of ASLAN003 or DMSO for 96 hours in the culture medium. Cells were collected, washed with 1 x PBS and staining with antibodies specific to human myeloid cell surface marker CD11b and CD14 for 30 minutes on ice. For primary AML or MDS BM cells, two additional antibodies specific to human CD13 and CD33 antigens were added. Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences, San Jose, CA). Only viable cells were included in the subsequent analysis of differentiation markers. These antibodies were purchased from Miltenyi Biotec (Germany) and their catalogue number are 130-091-240 (CD11b-PE), 130-113-143 (CD14-APC), 130-103-667 (CD13-FITC, clone: REA263), 130-101-054 (CD33-APC-Vio770, clone: AC104.3E3). The Hoechst 33258-Pacific blue (ThermoFisher Scientific, 3569) was mixed into each sample to separate the viable cells from dead cells. This blue fluorescent Hoechst dye is a cell-permeable nucleic acid stain which is very sensitive to recognize DNA damage. The gating strategy was optimized by using each antibody individually, in combination, together with their respective isotype control antibodies. BD FACSDiva 8.0 analysis software (BD Biosciences) was employed to analyse the data and generate FACS plots.

4. Cell viability assays

CellTiter-Glo® Luminescent Cell Viability Assay (CTG assay, Promega, Madison, WI) was used to determine cell growth and viability. In brief, twenty thousand viable leukemic cells were seeded in 96-well culture plates in 100 µl per well in triplicates and treated with compounds for 48 hours. Three independent experiments for each were performed. GraphPad Prism (version 7, San Diego, CA) was employed to calculate the IC_{50} values by fitting the raw data points with sigmoidal nonlinear regression and construct the dose-response curves.

5. Wright-Giemsa staining and NBT assay

Wright–Giemsa staining is commonly used to evaluate cellular morphology of bone marrow cells. In brief, cells were cytospinned on the slide and air dried. Covered spots on the slides were stained with Wright–Giemsa solution (Sigma WG16-500ML) for 1 minute, then washed and air dried. To determine NBT reduction, cells were incubated with 1 mg/mL NBT solution (Sigma N5514) and freshly diluted TPA (Tetradecanoylphorbol-13-acetate, Sigma, P8139) at 37°C for 30 minutes. Cytospin slides were prepared and counter-stained with 0.5% safranin (Sigma, HT90432) in 20% ethanol. In both of assays, the slides were examined with an Olympus IX71light microscope (Japan), and images were captured with a DP71 charge coupled device (CCD) camera. For NBT assay, 10 random 10x fields under the microscope were selected for counting the NBT positive cells containing precipitated formazan particles and negative cell numbers. The mean number of NBT positive cells was calculated as the percentage of NBT positive cells relative to the average of the total number of the cells counted for each sample.

6. Western blot analysis

Immunoblotting analysis was performed using standard techniques. Briefly, total protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) and SDS-PAGE and immunoblot analyses performed with different antibodies. The following antibodies used were: anti-ElF4B (D-4) (cc-376062) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-cleaved caspase-3 (Asp175) antibody (#9661), anti-cleaved caspase-7 (Asp198) antibody (#9491), anti-cleaved caspase-8 (Asp391) antibody (#9496), anti-c-FOS antibody (#4384) from Cell Signaling Technologies, Inc. (Danvers, MA), and anti-RPS6 antibody (ab70227) from Abcam (Cambridge, U.K.). HRP conjugated anti-GAPDH (Santa Cruz Biotechnology, SC-47724) was used for loading control.

7. Real-time quantitative reverse transcriptase-PCR (qRT-PCR)

To quantify c-FOS, c-JUN, FAS, CD68, and CBS gene expression, RNA samples were extracted from relevant cell lines and cDNAs were generated by using SuperScript® III RT (ThermoFish) with oligo-dT primer. qRT-PCR was performed using Power SYBR® Green PCR Master Mix was used as the recommendation by the manufacturer (Applied Biosystems). GAPDH was used as internal control. SDS 2.2.1 software (Applied Biosystems) was used to perform relative quantification of target genes using the comparative C_T ($\Delta\Delta C_T$) method. The primer sequences are provided in Supplemental Table S1.

Supplemental Table S1. Primers used in real-time quantitative (q) RT-PCR.

Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')
c-FOS	CTGGCGTTGTGAAGACCAT	TCCCTTCGGATTCTCCTTTT
c-JUN	ATCAAGGCGGAGAGGAAGCG	TGAGCATGTTGGCCGTGGAC
FAS	TGCAGAAGATGTAGATTGTGTGATGA	GGGTCCGGGTGCAGTTTATT
CD68	TCAGCTTTGGATTCATGCAG	AGGTGGACAGCTGGTGAAAG
CBS	GTTGGCAAAGTCATCTACAAGCA	GGGCGAAGTGGTCCATCTC
GAPDH	GTATTGGGCGCCTGGTCAC	CTCCTGGAAGATGGTGATGG

8. RNA-seq and data analysis

KG-1 and MOLM-14 cells were treated with either DMSO or ASLAN003 1μM or 500 nM respectively for 96 hours. Each treatment was performed in triplicates. Total RNA was extracted using the RNeasy Mini Kit following the manufacturer's protocol (Qiagen, Hilden, Germany). RNA quantity, quality, and purity were assessed with the use of the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA sequencing (RNA-seq) libraries were constructed by TruSeq Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer's instructions and subjected to Illumina Hiseq4000 deep sequencing (paired-end reads of 100 bases) at BGI (Shenzheng, China). The RNA-seq data were checked for raw sequence quality using FastQC v0.11.5, and were filtered to remove adaptor sequences, contamination and low quality read. The sequences were mapped to human genome hg38 using

STAR v2.4.2a and subsequently, transcript quantification using RSEM 1.2.25 with Gencode v24 annotation. All samples have average read length of 100 and unique mapping rate > 98% (average 46 million unique reads). EBseq v1.20.0 was used to identify differentially expressed genes.[25] T-test was computed by Matlab® R2012a, statistics toolbox version 8.0 (MathWorks; Natick, MA). Single-sample Gene Set Enrichment Analysis (ssGSEA) and Gene Ontology (GO) analysis conducted by R Bioconductor package GSVA 1.28.0.

9. Assessment of mitochondrial membrane potential (MMP)

Briefly, KG-1 and MOLM-14 cells were plated in a 6-well plate at a density of $1x10^6$ cells/well and treated with either DMSO (control), ASLAN003 1 μM, 2 μM for 48 hours at 37˚C. Cells were washed with PBS and put back in a 5% CO_2, 37°C incubator for 30 minutes, protected from light. Then, cells were centrifuged and resuspended in 1 mL of Assay Buffer, followed by assessing the fluorescence intensity using an LSRII flow cytometer (BD Biosciences) in the FL1 (green) and FL1 (red) channels. A decline in the ratio of red to green fluorescence implied a loss of MMP.

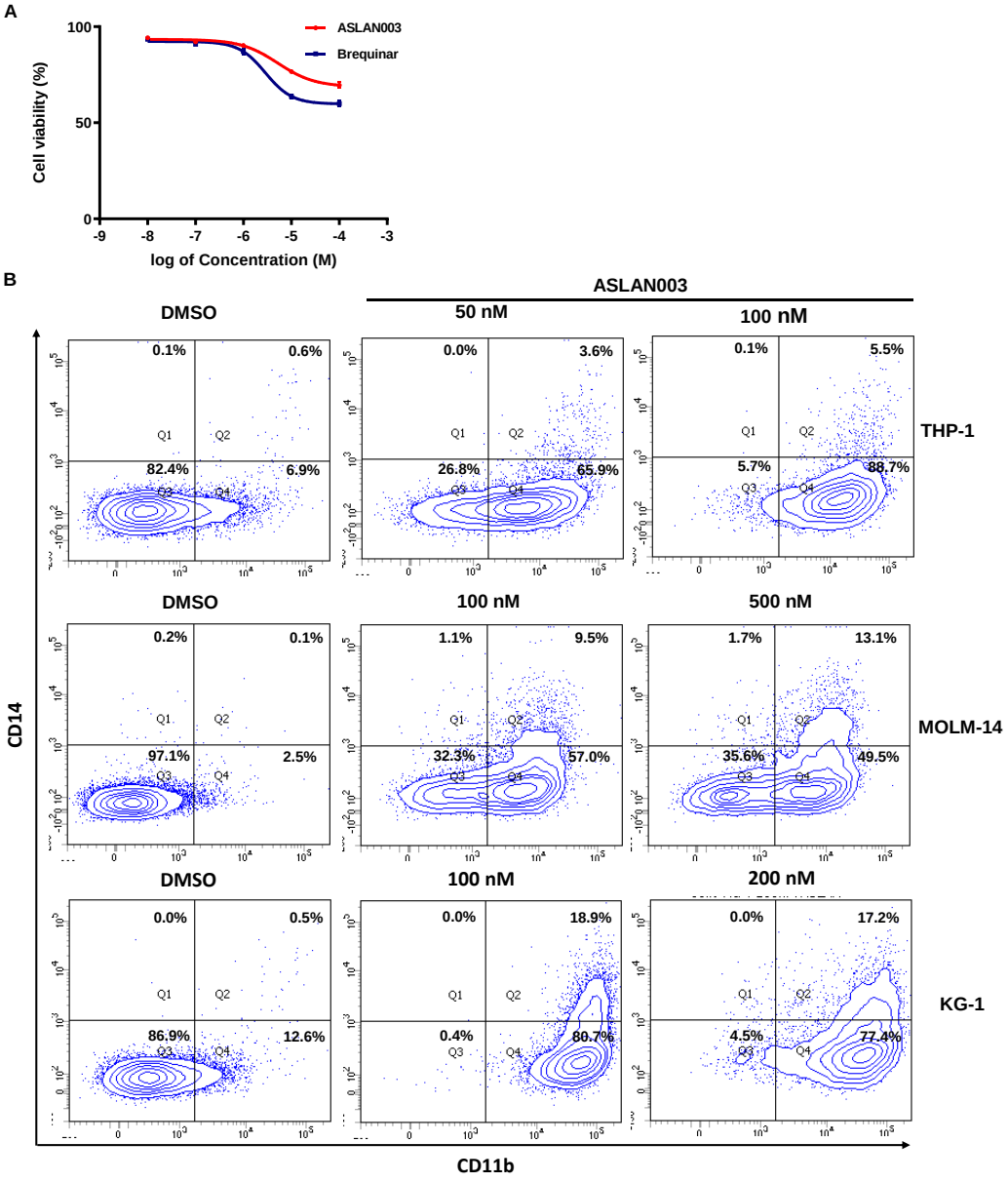
10. *In vivo* efficacy of ASLAN003

For THP-1 and MOLM-14 xenograft experiments, all the mice were closely monitored and weighed. Mice were sacrificed when the disease signs were observed, like hunched back, loose fur, sluggish response, etc, by CO_2 euthanasia. Detailed mouse necropsy was then conducted and tumour, liver, spleen were harvested. In 3-4 mice in each group, leukemic burden and differentiation status of leukemic cells were analysed 7 days post treatment. Mouse bone marrow was harvested from both femur and tibia of diseased mice and bone marrow mononuclear cells were separated by the Ficoll-Paque™ method (Sigma-Aldrich). Leukemic engraftment in bone marrow, peripheral blood, liver, spleen was analysed by human specific CD45 positive cells on an LSRII flow cytometer (BD Biosciences, San Jose, CA). Differentiation status was also assessed in the bone marrow and peripheral blood by human specific CD11b and CD14 markers.

Human specific anti-CD45 antibody was purchased from ThermoFisher (eBioscience™, clone HI30, #17-0459-42) and mouse specific anti-CD45 antibody was obtained from BD Biosciences (BD Pharmingen™, clone 30-F11, #553081). Human specific anti-CD11b and anti-CD14 antibodies were the same as those used in *in vitro* differentiation assays.

For PDX models, bone marrow cells were harvested from the AML-14 or AML-23 PDX line expanded in NSG mice and filtered through a nylon filter (35 µm) to obtain a single-cell suspension. After lysis of red blood cell (RBC), the cells were washed with PBS and spun down at 1500 rpm for 5 minutes. Cell count was performed using an automated hemocytometer, Celltac Alpha MEK-6450 (Nihon Kohden, Japan). The recipient mice (NGS) were subjected to sub-lethal irradiation (2 Gy) 48 hours prior to transplantation. An equal amount of whole bone marrow cells (3×10^6 cells) was transplanted to each mouse. Treatment was initiated 5 or 8 days post-transplantation. Engraftment of patient cells was assessed weekly by flow cytometry in a similar way as performed in human AML cell line xenograft modes.

Supplemental Figures



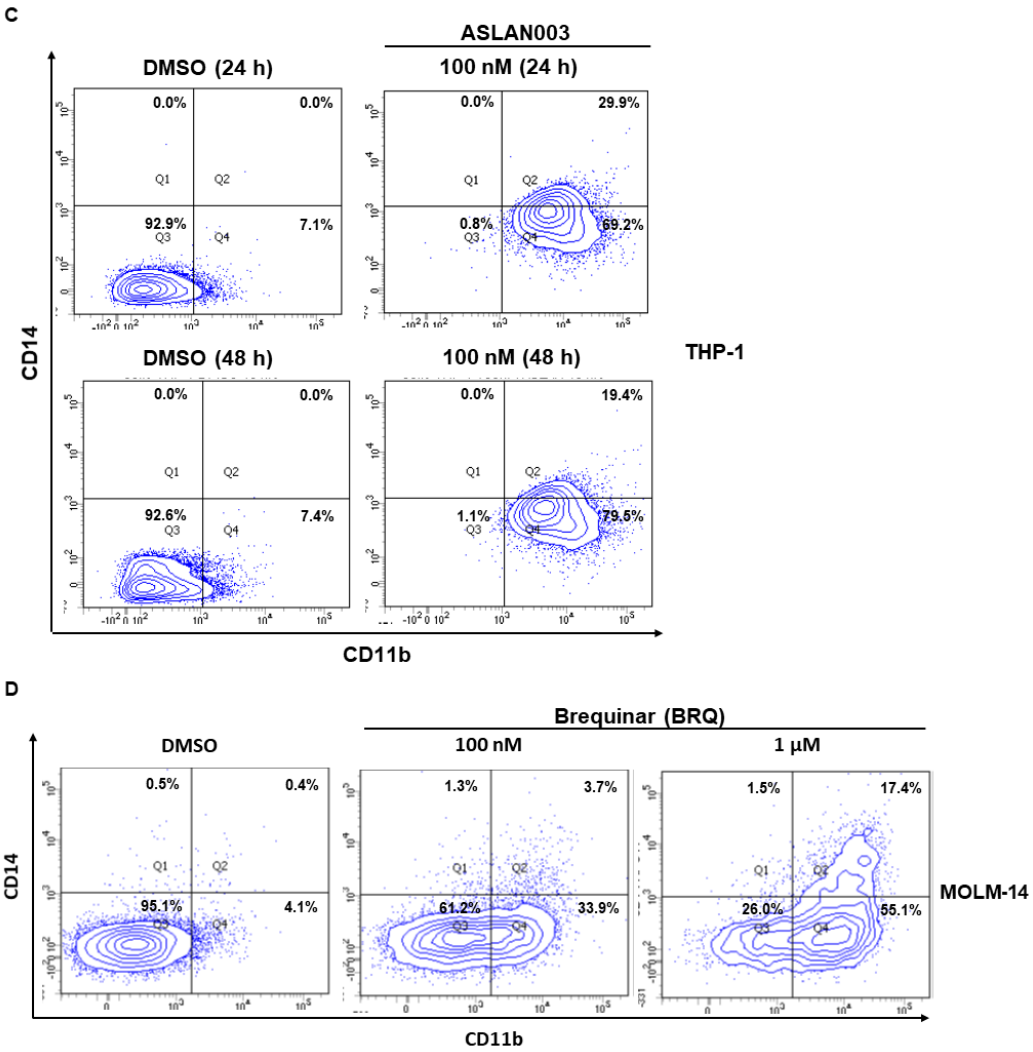


Figure S1. (A) Dose-response curves of ASLAN003 or brequinar treatment for 48 hours on cell viability of normal CD34+CD38+ myeloid progenitor bone marrow cells from healthy donors (n = 3). The percentage of cell viability relative to that of DMSO-treated cell is shown. Data represent 3 independent replicates (Mean ± SD). **(B – D) Original FACS plots of THP-1, MOLM-14 and KG-1 treated with DMSO control or ASLAN003 (B), time-dependent treatment on THP-1 (C) and MOLM-14 cells treated with DMSO or brequinar (D).**

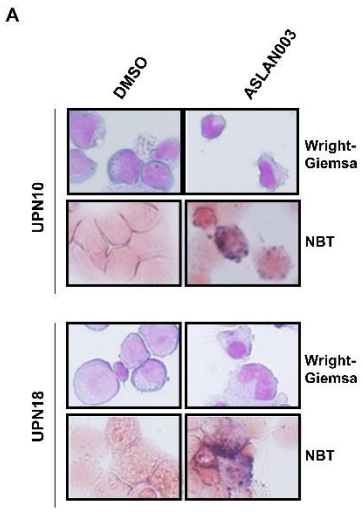


Figure S2. Representative images of Wright-Giemsa staining and NBT assay of UNP10 and UNP18 treated with DMSO and ASLAN003. The ASLAN003 dose was 1 μM for UNP10 and 4 μM for UNP18. The treatment time was 96 hours.

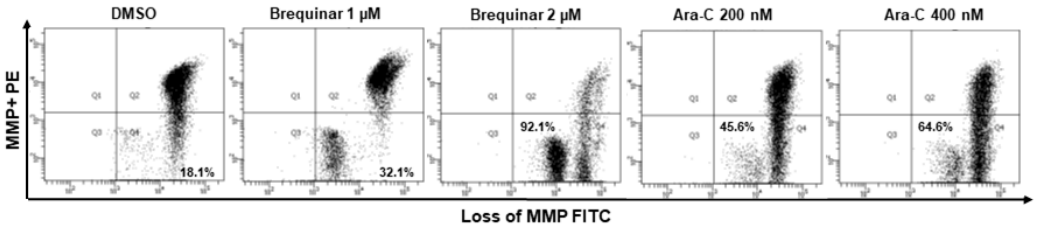


Figure S3. The effects of brequinar and Ara-C on mitochondrial membrane potential (MMP). MOLM-14 cells were exposed to Brequinar 1, 2 µM or Ara-C 200, 400 nM or DMSO as the control for 48 hours and then stained with JC-10 and analysed by flow cytometry for quantitation of intrinsic MMP ($\Delta\Psi m$). Representative FACS plots were shown.

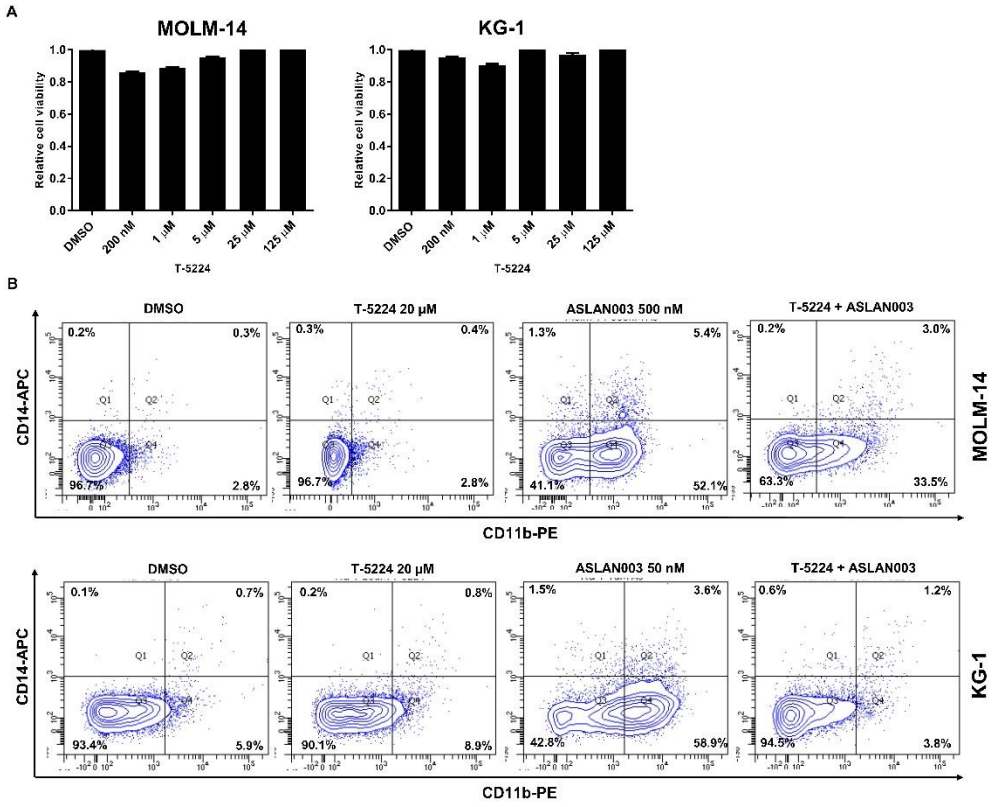


Figure S4. The effects of AP-1 inhibitor, T-5224 on cell viability and differentiation of AML cells. (A) MOLM-14 and KG-1 cells were treated with T-5224 at gradient concentration for 48 hours and the viabilities were measured using CellTiter-Glo (CTG) assays. The relative viabilities were normalized to cells incubated with DMSO (control). The figures represent mean ± SD (n = 3). **(B)** Representative FACS plots of MOLM-14 and KG-1 treated with DMSO, T-5224 alone, ASLAN003 alone and combination of T-5224 with ASLAN003. The treatment time was 96 hours.

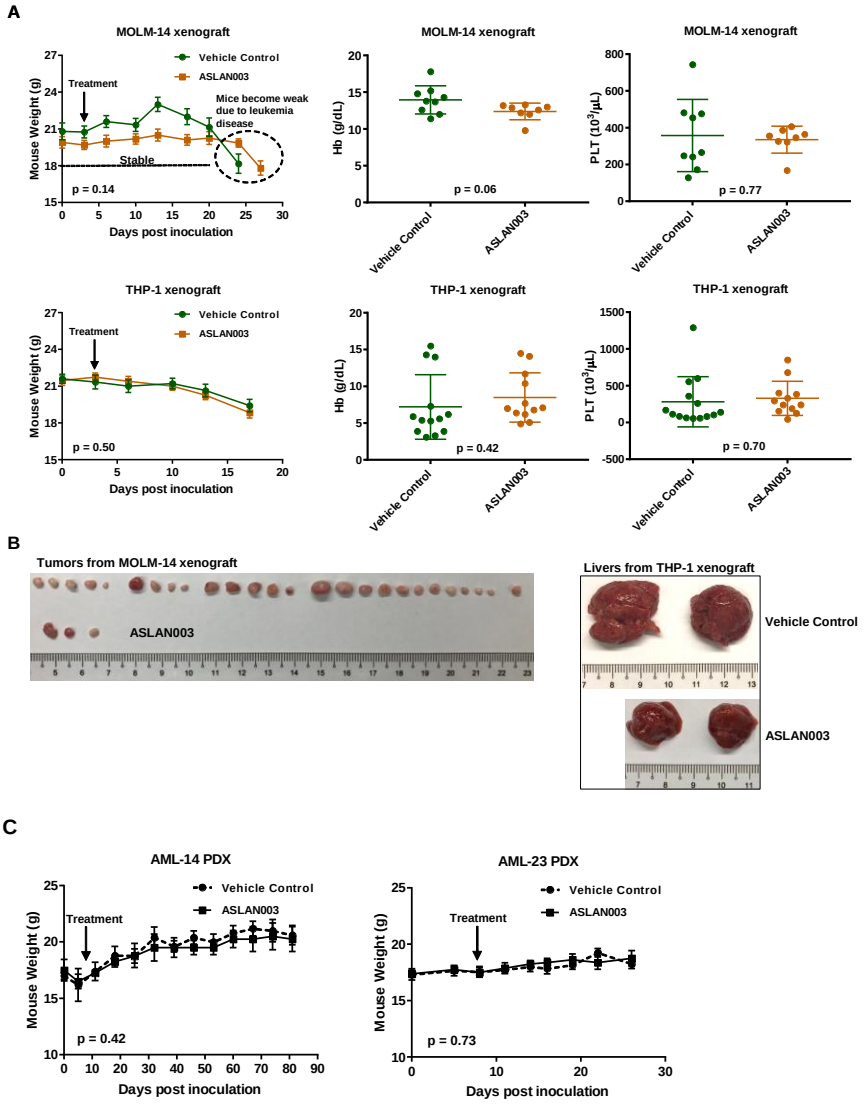


Figure S5. The *in vivo* efficacy of ASLAN003 in human AML xenograft models and PDX models. (A) Body weight (g, mean ± SD) of mice in MOLM-14 and THP-1 xenograft models during the experiment course. There is no statically difference in the body weight between ASLAN003-treated mice and vehicle-treated mice (p = 0.14 for MOLM-14 xenografted mice and p = 0.5 for THP-1 xenografted mice). ASLAN003 didn't alter the hemoglobin concentration and platelet count in these two xenograft models tested as demonstrated in dotplots with mean ± SD. The p values were sketched in each graph. **(B)** Representative images of disseminated leukemic tumors harvested from one mice in vehicle and ASLAN003 group of MOLM-14 xenograft model

were shown. Representative images of livers harvested from two ASLAN003-treated mice and two vehicle-treated mice in THP-1 model were presented. **(C)** Body weight (g, mean ± SD) of mice in PDX AML-14 (n = 5 for control and 4 for ASLAN003-treated group) and AML-23 xenografts (n = 9 for control and 8 for ASLAN003-treated group) during the experiment course. There is no statically difference in the body weight between ASLAN003-treated mice and vehicle-treated mice ($p = 0.42$ for PDX AML-14 xenografts and $p = 0.73$ for PDX AML-23 xenografts).

Supplemental Table S2. Common gene expression changes of KG-1 and MOLM-14 cells treated with DMSO and ASLAN003 (RNA-seq data).

KG-1 & MOLM-14_Up Regulated	KG-1 & MOLM-14_Down Regulated
CR1	NRARP
S100A12	PDSS1
DES	RPL24
HK3	RPL36A
FAM65B	RPL13A
ANKRD22	SLIRP
SERPINB2	PDCD11
ADGRE4P	MRPL45
ADGRE1	EXOSC5
LRRK2	MDN1
PTAFR	ZNF436
SLPI	GNB2L1
LGALS3	DARS2
MYOF	BOP1
CD101	WT1
BPI	TUBA1C
NCF2	MRRF
PECAM1	DUS2
OLIG1	PSAT1
S100P	RPL9P9
COL24A1	RPL7A

MPEG1	TMEM209
SORT1	SLC25A6
LYZ	RPL7AP6
CYBB	RPL10A
CECR1	TYRO3
IL1B	POLR2H
ANXA8	SNHG7
ITGAM	SPDL1
FGL2	GPRC5C
PILRA	RPLP2
TNFSF10	GTPBP3
CD86	RPL23A
TGM5	EBPL
SMIM14	IRX3
SEMA4A	NOA1
FBP1	HMGB3
CD180	PRR11
CD36	DDX49
CD84	C19orf48
S100A4	RPS29
SPINT2	OXCT1
CYP1B1	RPS5
SEMA6B	CTD-2619J13.14
ANXA1	GPT2
SRD5A1	THG1L

CFP	RP11-620J15.3
IPCEF1	ARHGAP11A
FGR	TOMM7
STARD5	RPL34
HLA-DRB1	MCAT
PHLDA1	RPL17
SLC44A1	KNOP1
KLF4	UBA52
ITGB7	SNHG4
FYB	RPS18
SLC2A6	RPL27
RBM47	RPL9
TIRAP	LZTFL1
LHPP	SLC7A1
SYT11	MRPS2
PTPRO	RPS9
JUN	RPL41
DAPP1	CLN6
ACPP	RPL37A
ST3GAL6	RPL35A
GAPT	RPL19
LRRC25	SLC16A1
GAS7	EEF1B2
IL16	RPL8
NLRP12	WDR4

RAB7B	FRA10AC1
SLC22A4	RPS7P1
CD300C	RPL3P4
CD244	SLC19A1
MEFV	RPL7
RIN3	RP5-940J5.9
NOD2	PTCD2
LY86	RPS7
C10orf54	DZIP1
SERPINB1	MIPEP
GPR35	MRTO4
RGS18	EEF1G
SLC43A2	RPL36
OSCAR	C10orf2
ITGB2-AS1	AGBL5
MS4A6A	RPS20
TYROBP	RPS17
KCNQ1	TUBE1
GBP2	CCNB1IP1
SERPINB8	NDC1
DHRS9	IPO5
GSAP	TFRC
SLC37A2	RPS3
TRIM58	RPL5
FAM43A	LRRC20

COL9A2	RPLP0P6
HCST	HMGA1
S100A6	RPLP1
SAT1	UTP20
STX11	RPL23
PAQR8	RFXAP
HLA-B	SLC38A5
NHSL2	SLC38A1
CD68	NAT8L
TMSB4X	SLC7A5
CSF2RA	EIF4B
C3	RPA3
DENND2D	RPS13
PLB1	RP11-371A22.1
C7orf55	RPS10
GPR183	ZBED3
LILRB4	MRM1
FAM49A	SNHG8
TFEC	RPLP0
SQRDL	RPS6
VASH1	ZNF395
S100A10	RPS28
TBC1D2	RPL10
SH3BP5	EPB41L4A-AS1
IL17RA	RPS19

U2AF1L4	RP1-239B22.5
ST8SIA4	RPS3A
GPCPD1	NCR3LG1
DPYD	RP11-475C16.1
RP11-1319K7.1	POLA1
FBXL5	RPS27A
RAB31	CENPV
MTMR11	NPM3
C19orf38	MATK
CXCR4	EIF3L
GLIPR1	NEDD4L
THBD	RPL6
MAP4K1	EEF1B2P3
SLITRK4	PES1
FCGR2A	PKN3
TRIM22	BMP2
RHOU	HNRNPA1
RNASE6	PHGDH
TIMP2	SNHG1
CYBRD1	RPS14
IDUA	RPL13AP5
IL1RN	LDHA
TPM4	RPS16
SLA	MIR9-3HG
PPP1R3F	RPL26L1

SAMHD1	RPL37
FGD4	FAM72D
MNDA	PACSIN3
MOB3C	RPL18
TMEM170B	GCAT
ZCCHC24	MZT2A
IRF5	NEFH
MXD1	CEP290
SCUBE1	PLPPR3
COCH	UAP1
CEP19	PTPN13
CCPG1	RP11-641D5.1
FRY	RPL7P9
TNFSF13B	CYB561
CPPED1	CENPH
ZNF467	KIF9
NINJ2	RP11-408P14.1
CTSL	RPS21
FIBCD1	HNRNPM
AP1S2	FAHD2B
PLD3	RPS3AP6
CST7	FAM72A
P2RY6	NLE1
TLR2	RPL18AP3
SLCO3A1	RPS12

KIAA0513	RPL6P27
CTSO	RPS3AP26
NCF1B	HNRNPA1P48
CCR1	HNRNPA1P7
PTGS1	RP11-161H23.5
TGFBR1	ELANE
BTG2	EFNB1
C1orf162	LDHAP4
ARSD	RP11-574K11.24
GNG7	CYB5D1
ZFYVE16	LRRC75A-AS1
GSN	RPL18A
HOOK3	DEPDC7
LRP12	WDR54
NFE2	U2AF1L5
ACER3	PRKCQ
TBC1D8	PRR5
DDX60L	WNT7B
MCTP1	GAS5
MIR4435-2HG	USP54
CYSLTR1	DDIT4
EEPD1	SNHG3
HCK	DANCR
IL4R	AK4
METTL9	FAAP24

NKG7	FAM72C
RRAGB	VAT1L
ACSL1	DLEU2
ASAH1	ASIC1
OSBPL11	GATM
JAK2	PRKCQ-AS1
FAM20C	SNHG5
SNX20	MAPK11
RPS6KA2	FBL
IL13RA1	GPR63
SH3BGRL3	BNIP3
BBS2	FAM72B
DENND1B	SLC25A25-AS1
COQ10B	CDPF1
MBOAT7	OLMALINC
CD300LF	ELOVL6
ATP10D	TERT
CPNE8	DDN
ARL6IP5	RP11-864I4.1
KLF13	FJX1
RIT1	DPY19L2P2
ADGRL3	GAL
ARHGDIB	TBC1D30
IGFLR1	MPO
CPQ	LGALS12

S100A11	CBS
GALC	CAMKV
CTSZ	
TBXAS1	
SCARB2	
IL12RB1	
LILRA2	
B3GLCT	
FCGR1A	
MILR1	
SUSD1	
TNFSF14	
GNS	
CKAP4	
CTSD	
CD74	
CYSTM1	
JAK2	
APH1B	
CHST13	
LCP2	
PAK1	
CES1	
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MYL12A	
HECA	
SMAD7	
INPP4A	
LAPTM5	
ITPK1	
CPM	
SLC12A6	
TANK	
TAGAP	

PNPLA8	
SDCBP	
CYLD	
KLHL2	
TGFBR2	
CCNG2	
TYMP	
GLIPR2	
TPST2	
AIF1	
INPP1	
C18orf32	
EPB41L3	
LST1	
RHOG	
HK1	
RHBDD2	
C16orf70	
UBE2D4	
EMILIN2	
CHM	
TUBA4A	
RNF130	
MSRB1	
SCPEP1	

HIPK3	
EPHA1	
ZYX	
PCTP	
FAS	
RFFL	
EVI2B	
RSRP1	
FNDC3B	
PSAP	
MBP	
LGALS1	
SLC22A15	
ENTPD1	
PITPNC1	
CSGALNACT2	
NADK	
OSTF1	
RASSF2	
SAMSN1	
ATP6AP2	
TOR1AIP1	
TAF8	
MKNK1	