The novel Isatin analog KS99 targets stemness markers in acute myeloid leukemia

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SUPPLEMENTARY

METHODS

AML Cell Lines

The human acute myeloid leukemia (AML) derived cell lines, HL-60, MV4-11 and KG-1 were obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, USA. All other cell lines were kindly gifted according to the acknowledgements. MV4-11 and U937 stably expressing the firefly luciferase (Luc2) with a fluorescent protein YFP (MV4-11) (1) or tdTomato (U937) (a gift from Kazuhiro Oka; Addgene plasmid #72486, Watertown, MA) were used for animal studies. C1498 cells were purchased from ATCC (Gaithersburg, MD) and were transduced with viral particles containing pUltra-LUC-Chili (Addgene plasmid #48688) and polybrene (8 µg/ml) 3 times every 12 hours. Cells were allowed to expand and sorted for tdTomato expression by flow cytometry. For all the cell lines, low-passage stocks were used and cultured for less than two months. MOLM-13 and MV4-11 were maintained in IMDM (Corning, Manassas, VA). U937, U937-Luc, OCI-AML2, HL-60, HL-60/VCR, KG-1, C1498-Luc cells were maintained in RPMI 1640 (Corning, Manassas, VA). HL-60/VCR1 cells were maintained in the presence of 1 µg/ml vincristine. Media contained 10% Heat Inactivated Fetal Bovine Serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) or 20% FBS (for KG-1) and 1% Penicillin and Streptomycin (GIBCO, Maryland, MD).

AML patient and healthy donor cells

Mutational profiling was performed as previously described in detail (2). Cell viability was ~80-95% post-thawing. AML with Myelodysplastic syndrome (MDS)-related changes were noted if there was a history of prior MDS or cytogenetic abnormalities associated with MDS as defined by the Vardiman group in an update to the WHO classification (3). Primary human AML cells were cultured in the serum-free medium StemSpan SFEM™ (Stem Cell Technologies, Vancouver, BC, Canada) and supplemented with recombinant human stem cell factor (SCF), Interleukin 3 (IL-3), FMS-like tyrosine kinase ligand (FLT3L), Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage-colony-stimulating factor (GM-CSF). All cytokines were purchased from Shenandoah Biotechnology, Warwick, PA. StemRegenin1 (SR1), (ChemieTek, Indianapolis, IN) and UM729 (Selleckchem, Houston, TX) were added to maintain the "stemness" of primary cells for stem cell assays to test LSCs (4). All cultures were supplemented with 1% penicillin/streptomycin and stored in a humidified 37˚C incubator with 5% CO_2.

Cell viability and Annexin V assay

Cells were plated at a density of $2x10^5$ to $4x10^5$ cells/mL and treated with increasing concentrations (10 nM-10 µM) of KS99 for 48 hours. Cell counts, and viability was determined using MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). The absorbance at 490nm was recorded using a plate reader. The background absorbance was subtracted from the data, and cell viability was normalized to control wells, containing only DMSO. For the apoptosis, Annexin V and/or 7AAD positive cells were detected by using Muse Annexin V & Dead Cell Kit (MCH100105, Millipore, Burlington,

MA). Data were collected by flow cytometer Muse Cell Analyzer (Millipore, Burlington, MA). The half maximal inhibitory concentration (IC_{50}) values were calculated by non-linear regression analysis using GraphPad Prism software. For the synergy studies, OCI-AML3 and MV4-11 cells were treated with Ara-C (0.062-4 μM) and KS99 (0.03125-2 μM) for 72 hours, and the combination index (CI) was calculated by CalcuSyn (5).

Colony-Forming assay

Cryopreserved human AML patient samples and cord blood mononuclear cells were cultured in triplicate in 12-well plates at a density of $0.1x10^5$ to $2x10^5$ cells per well in Human Methylcellulose Complete Media (R&D Systems, Minneapolis, MN). Plating densities were selected for each case to yield colony out-growth of 20-100 colonies per well. Human AML cell lines were cultured at a density of 250-500 cells per well in Human Methylcellulose Base Media (R&D Systems). During the assay set-up, cells were added simultaneously to the culture media with KS99 or controls. Colonies were propagated for 10-14 days, and blast colonies (>20 cells/colony) were counted in a blinded manner under the light microscope.

Chemical reagents

Cytarabine (NDC 61703-305-38) was purchased from Penn State Hershey Medical Center Pharmacy. Ibrutinib was purchased from ChemieTek (Indianapolis, IN). Vincristine was purchased from Sigma-Aldrich (St. Louis, MO). KS99 was synthesized by Dr. Amin's group, Penn State College of Medicine Organic Synthesis Core.

Flow Cytometry

To detect pSTAT3 in LSCs, cells were first stained against cell surface markers followed by fixation, permeabilization, and intracellular staining. For cell surface staining the procedure described above was followed, anti-human CD34-BV421 (BD Biosciences, Franklin Lakes, NJ) was used. After cell surface staining, cells were fixed with pre-warmed fixation buffer for 20 minutes at room temperature in the dark, washed and exposed to Intracellular Staining Perm Wash Buffer (BioLegend) by centrifuging three times for 5 minutes, followed by anti-STAT3 phospho (tyr705) antibody (BioLegend) staining for 20 minutes at room temperature in the dark. Post exclusion of debris and dead cells, pSTAT3 was detected in LSCs ($CD34^+CD38^-$).

Western Blotting

Whole cell lysates were harvested in 100 μL 1X RIPA buffer (20mM Tris-HCL (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 30 mM sodium fluoride, and complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany)). Lysates were centrifuged (20,000 x g) for 15 min at 4°C to remove cell debris. Total protein concentrations were quantified using the BCA assay from Pierce (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of total denatured protein were resolved by NuPAGE 4-12% Bis-Tris gel electrophoresis (Life Technologies Carlsbad, CA, USA) and transferred to PVDF membranes (Life Technologies Carlsbad, CA, USA). Membranes were blocked for 1 h at room temperature in 5% milk/TBS-T, incubated overnight at 4°C with primary antibodies (1:1000), and immunodetection was done with corresponding secondary IgG HRP-linked antibodies (1:5000) using the ECL chemiluminescence reagents (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Antibodies against various proteins were obtained

from the following sources: STAT3, phospho-STAT3 (Tyr705), BTK, phospho-BTK (Tyr223) (Cell Signaling, Danvers, MA, USA); GAPDH, ALDH1A1 (Santa Cruz Biotechnology, Dallas, TX, USA). The goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugates and goat anti-mouse IgG-HRP conjugates were purchased from (Cell Signaling, Danvers, MA, USA).

Flow cytometry

To detect apoptosis in LSCs, DMSO or KS99-treated cells were washed and stained with APC-Cy7-labeled anti-human CD45, and either with anti-human CD34-FITC, anti-human CD38-APC, anti-human CD123-APC, anti-human TIM-3-PE-Cy7, or anti-human CD96-BV711 monoclonal antibodies for 30 minutes on ice, washed with staining buffer and stained with Annexin V-BV421 and 7AAD to detect apoptosis. All the antibodies were purchased from BioLegend, San Diego, CA. Forward and side scatter gating was used to exclude the debris. CD45-positive $CD34^{+}CD38^{-}$, $CD123^{+}$, $TIM\text{-}3^{+}$ or $CD96^{+}$ cell population was used to identify LSCs.

ALDH assay

Cells treated with either DMSO or KS99 for 48 hours were washed with staining buffer and suspended in ALDEFLUOR assay buffer. Diethylamino benzaldehyde (DEAB), a broad inhibitor of ALDH was used as a control to detect the background fluorescence. Cells were exposed either to ALDEFLUOR DEAB or ALDEFLUOR reagent for 30 min at 37°C. All flow cytometry data were collected by using multicolor flow cytometer LSRII (BD Biosciences) and FACSDIVA software (BD Biosciences), and analyzed by FlowJo software (FlowJo LLC, Ashland, OR).

***In silico* docking of KS99 with ALDH1A1, BTK, and STAT3**

The crystal structure of human ALDH1A1 and BTK was obtained from Protein Data Bank (PDB ID: 4WJ9 and 6DI1 respectively) while that of human STAT3 isoform 1 protein (1BG1.pdb) was developed using Swiss-Model with residues ranging from position 136-715 and showed 99.83% sequence identity. The KS99 structure was prepared using ChemDraw software and converted into PDB using Open Babel. Energy minimization was done using YASARA server (6).

Animal Studies

The Penn State College of Medicine Institutional Animal Care and Use Committee (IACUC) approved of all animal procedures.

Bioluminescence imaging (BLI):

Luciferase-expressing U937 (U937-Luc), MV4-11 (MV4-11-Luc) or C1498 (C1498-Luc) cells-bearing animals were imaged using the IVIS Lumina LT Series III imaging system (Perkin Elmer, Waltham, MA) 7–10 min after D-luciferin (Gold Biotechnology, St Louis MO) was injected intraperitoneally (150 mg/kg). Bioluminescence images were taken from ventral sides of Isofluorane-anesthetized mice. The photons emitted from cells expressed as total flux

(photons/s/cm^2/steradian), were quantified and analyzed using the "Living image" software (Perkin Elmer).

Pharmacokinetics (PK) studies:

NOD. Cg-Prkdc-scid Il2rgtm1Wjl/SzJ (NSG) (n=3) mice (The Jackson Laboratory, Bar Harbor, ME) were intraperitoneally injected with 2.5 mg/kg body weight of KS99, animals were euthanized, and blood was drawn by cardiac puncture at various time points. Samples were kept at room temperature for 30 minutes followed by plasma separation by centrifugation for 5 minutes at 5,000 rpm. KS99 was extracted from plasma. Briefly, 20uL plasma was mixed with 40uL acetonitrile/water/formic acid (90/10/0.1, v/v/v) and vortexed, and then the mixture was centrifuged for 10 min at 40°C at 8,765 g. The supernatant was transferred to the autosampler vials before loading onto UPLC (Waters)/MS/MS system (ABSciex 4000 Q Trap).

Efficacy studies:

Human AML U937-Luc cells (1 x 10^4) were engrafted by intravenous (IV) injection into a 6-8-week old NSG or NOD.Cg-Rag1tm1Mom Il2rgtm1Wjl/SzJ (NRG) (The Jackson Laboratory) mice. Animals were randomized into treatment groups on day 4 either based off on animal body weights or total body Bioluminescence Imaging (BLI) signals. Beginning on day 5 (post-engraftment) mice were treated through an intraperitoneal (IP) injection with either vehicle control (DMSO), KS99 (2.5mg/kg), Cytarabine (Ara-C, 50mg/kg) or combination of KS99 and Ara-C every other day for 2-4 weeks. The study was terminated after the final treatment, and mice were sacrificed. Bone marrow cells were harvested, and leukemic tumor burden was evaluated by staining with APC-Cy7-labeled anti-human CD45 (304014, BioLegend) and dead cell exclusion dye, 7AAD. Data were collected by flow cytometry using a BD LSR II flow cytometer.

For syngeneic mouse AML model, murine leukemia C1498-Luc cells ($2x10^6$) were injected via IV route into albino C57BL/6J mice (The Jackson Laboratories). Animals were randomized based off of BLI signals and treated as indicated in supplementary figure 3D. Kaplan–Meier analysis was conducted to compare survival curves between vehicle control, KS99 (2.5mg/kg), Ara-C (50mg/kg) or combination-treated mice.

For the subcutaneous xenograft model, $2.5x10^6$ MV4-11-Luc cells were implanted subcutaneously into the right flank of healthy female NSG mice. Treatment was initiated on day 7, once visible tumors were established in all the animals. KS99 (2.5mg/kg) was delivered through IP route three times a week for three weeks. A control group received an equivalent volume of DMSO. Mice were weighed weekly throughout the study. At the termination of the study, animals were euthanized, and tumors were isolated and weighed. Tumor growth inhibition was assessed by comparison of the mean change in tumor weight for the control and treated groups.

Statistical analysis

GraphPad Prism 6.0 software (GraphPad Software, CA, USA) was used for the statistical analysis. Data were subjected to the Chou-Talalay method for determining the combination index using CalcuSyn software (Bio-Soft, Cambridge, United Kingdom) and combination index (CI) values plotted against fraction affected. Using this approach, combination index values of < 0.9 are synergistic, >1.1 are antagonistic, and values 0.9–1.1 are nearly additive. An unpaired t-test was used to determine the significance of differences between two groups, and one-way analysis of variance (ANOVA) was used to estimate the differences between three or more groups. All data are expressed as means ± Standard Error of the Mean (SEM) and are representative of at least two-three experiments. P values <0.05 (95% CI) are considered statistically significant. Sample sizes and a number of times experiments were repeated are indicated in the figure legends. Number of asterisks in the figures indicates the level of statistical significance as follows*: *P*<0.05, **P*<0.01, ***P*<0.001, ****P*<0.0001.

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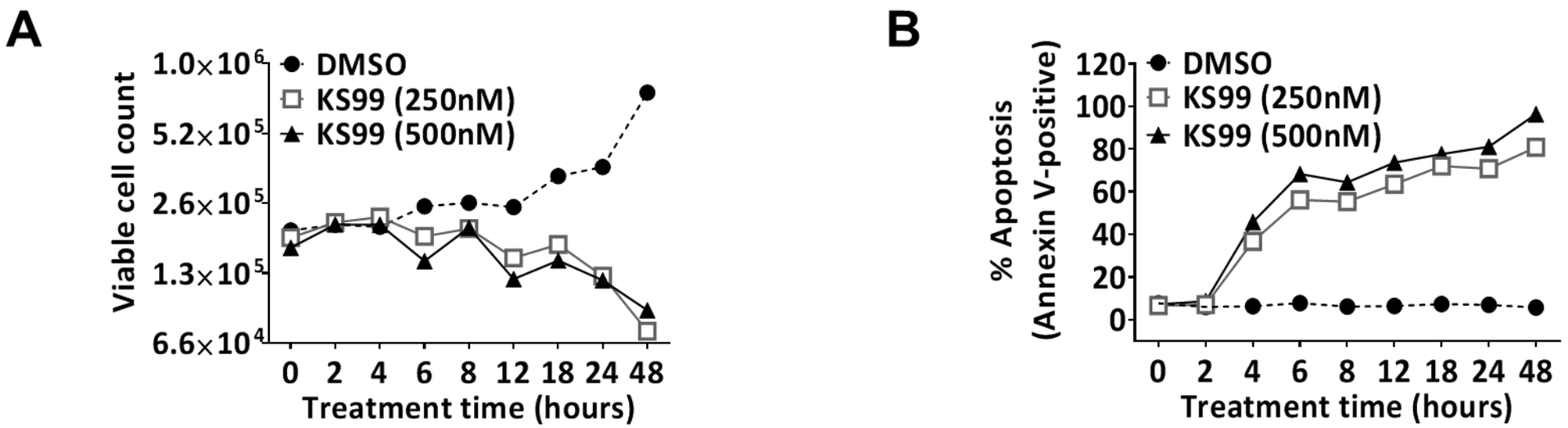
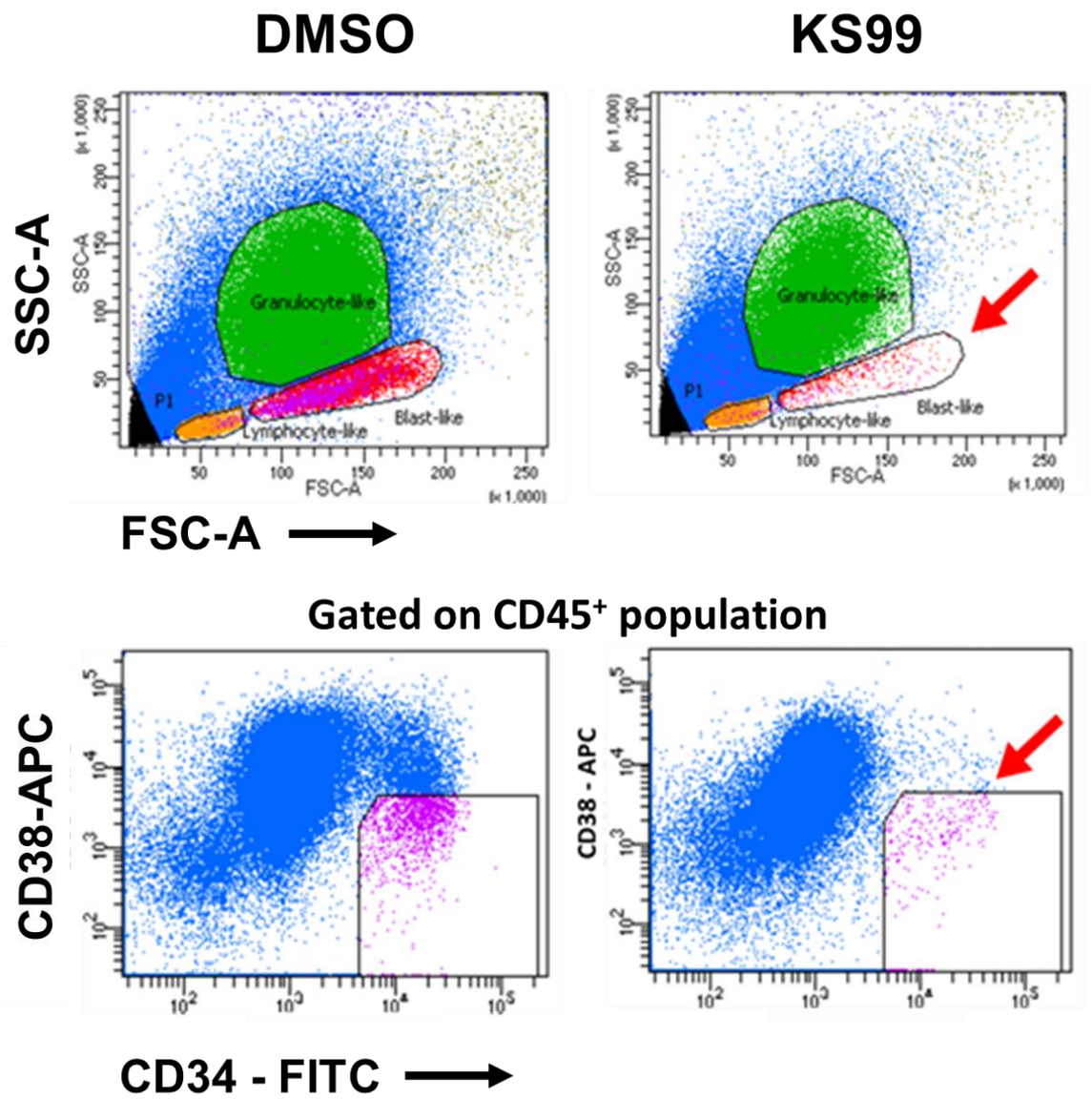
Supplementary Figure 1.



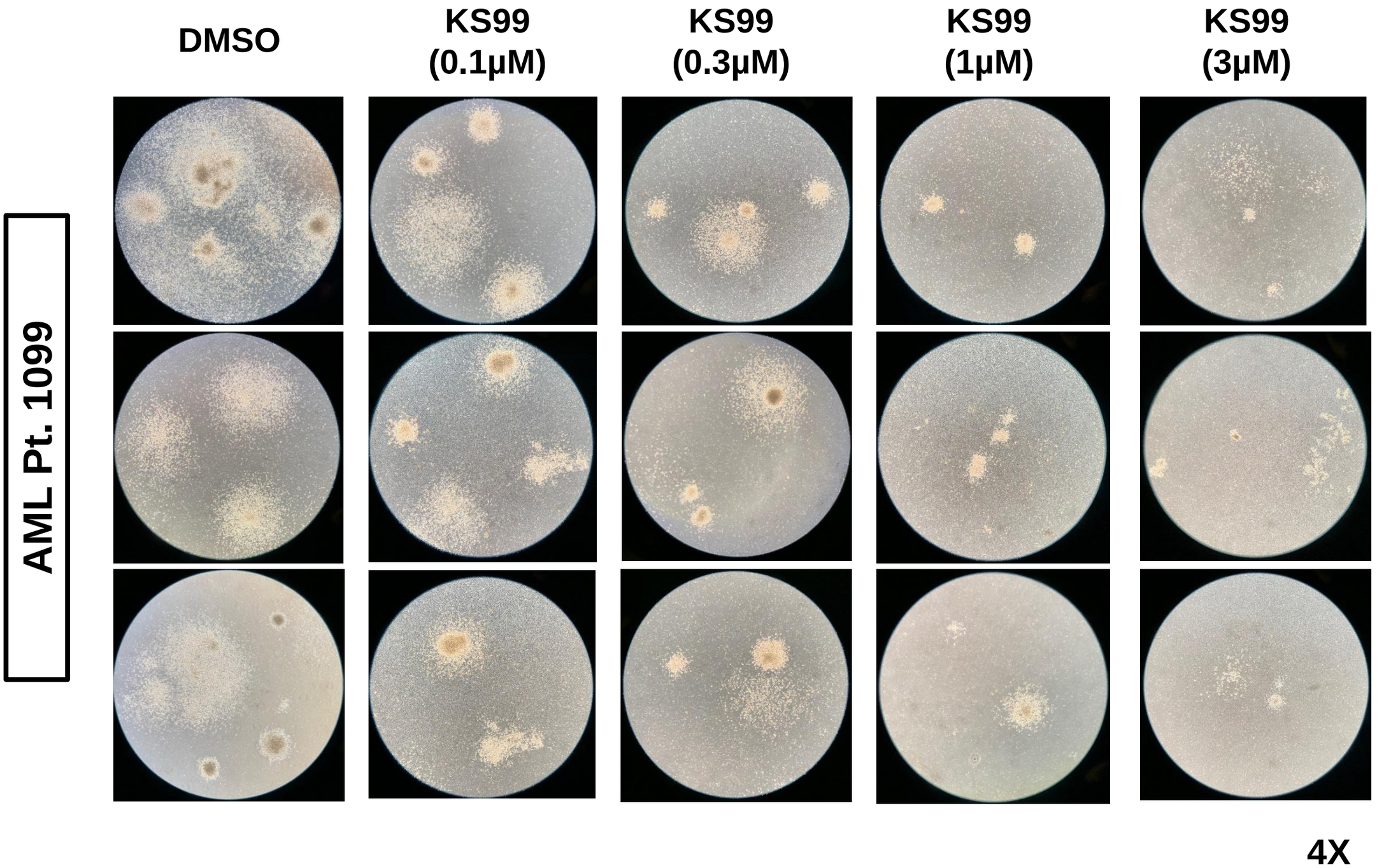
Supplementary Figure 1. (A-B) Time dependent activity of KS99 in MOLM-13 cells. MOLM-13 cells were treated with vehicle control (DMSO) or KS99, and cell viability (A) and apoptosis (B) were determined at various time points. (C) Decrease of Ara-C IC50 for OCI-AML3 and MV4-11 cells when combined with increasing concentrations of KS99.

Supplementary Figure 2. Induction of apoptosis in LSCs after treatment with KS99



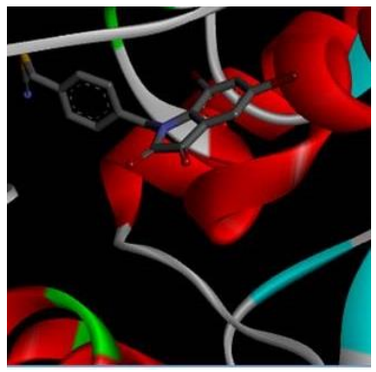
Supplementary Figure 2. Data shows effect of KS99 (3µM) on blast-like cells (upper panel, red arrow) or LSCs (lower panel, red arrow) relative to other cell populations in the AML patient sample.

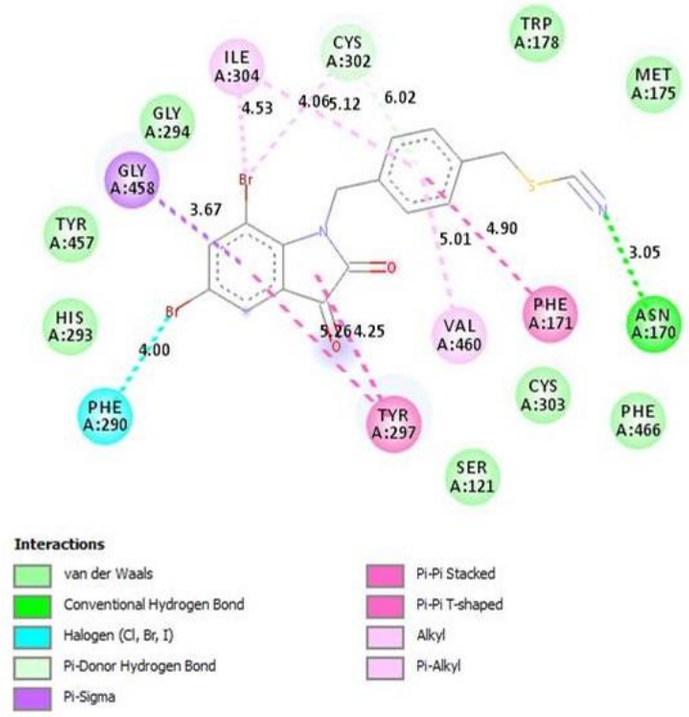
Supplementary Figure 3. Representative microscopy images (4X) of AML Pt. 1099 colonies after KS99 treatment



Supplementary Figure 4. Docking analysis

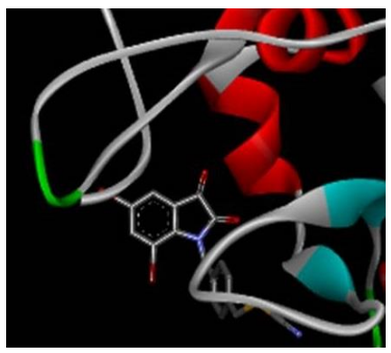
A

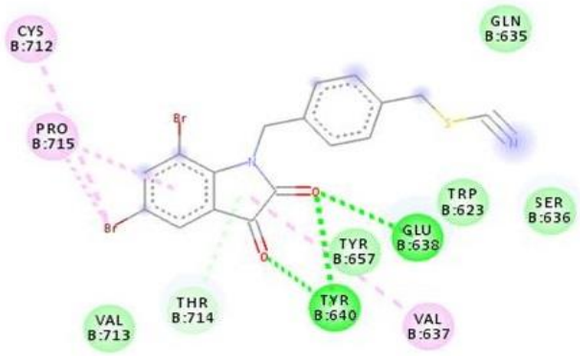




2D docked conformation of ADLHA1-KS99 complex

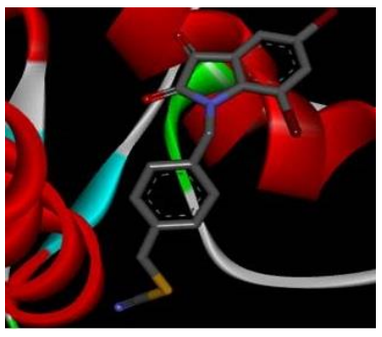
C

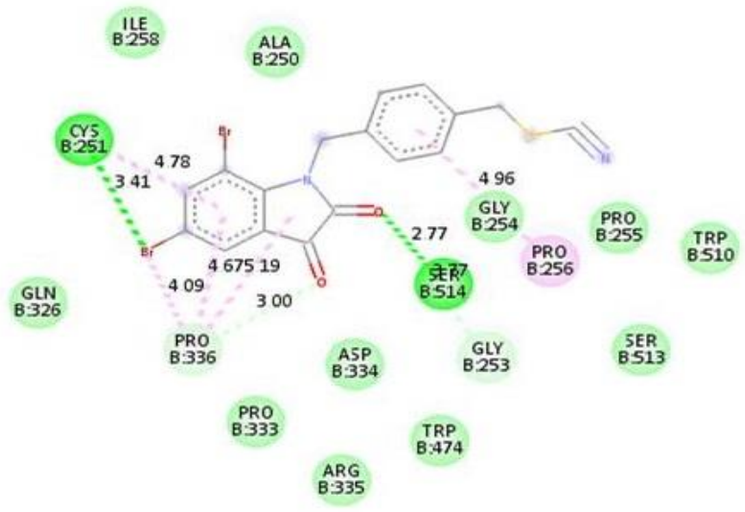




2D docked conformation of phosphotyrosine pocket of STAT3-KS99 complex

B

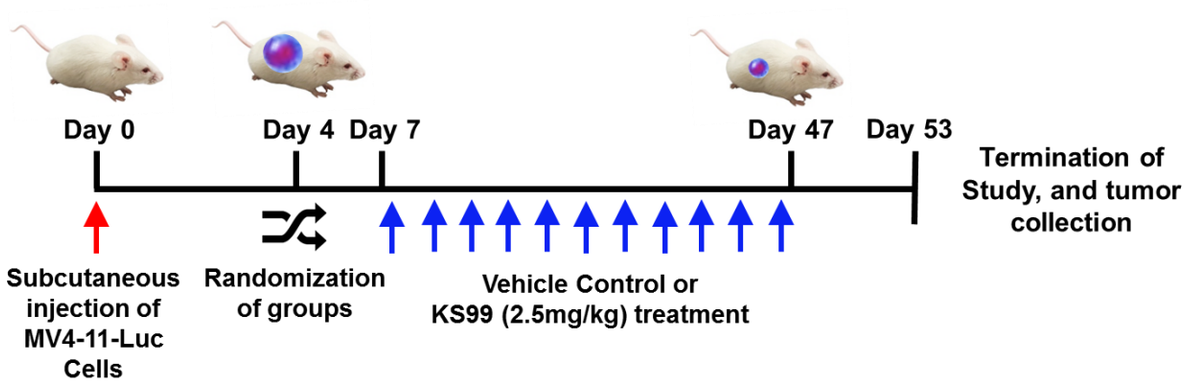




2D docked conformation of STAT3-KS99 complex

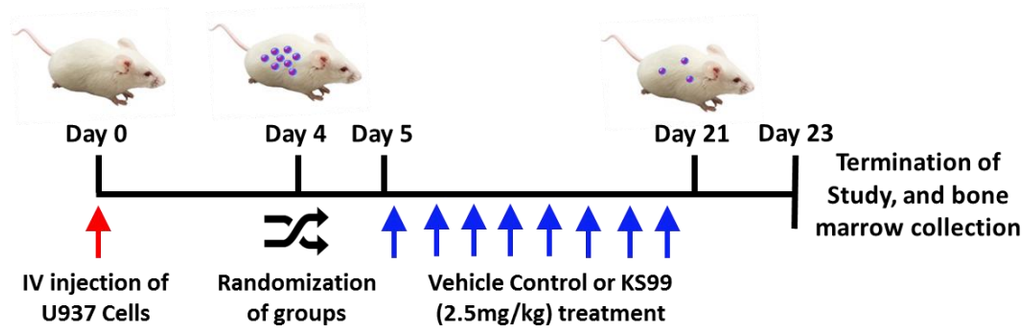
Supplementary Figure 5. Experimental scheme for animal efficacy studies

A



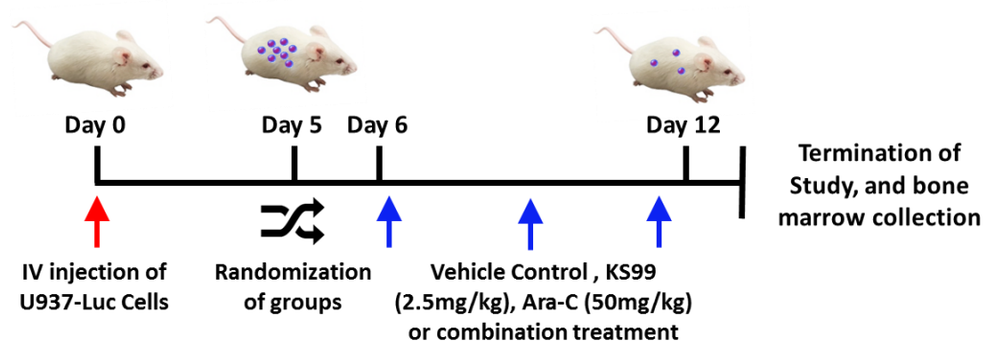
Subcutaneous MV4-11-Luc in NSG mice

B



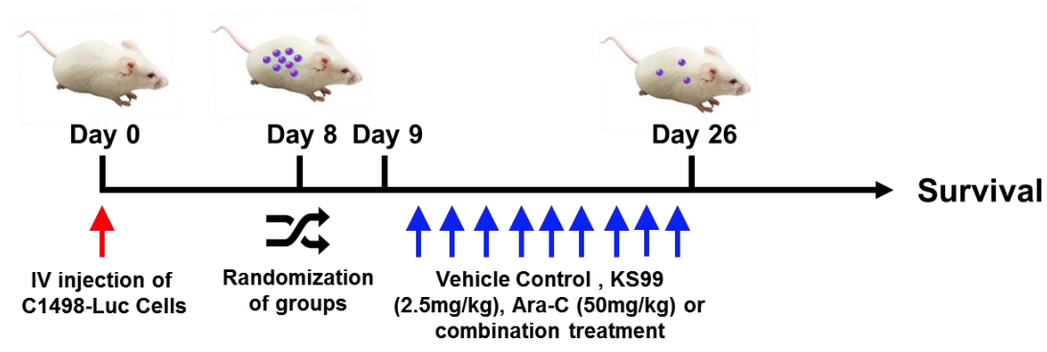
Disseminated U937 in NSG mice

C



Disseminated U937-Luc in NRG mice

D



Disseminated C1498-Luc in albino C57BL/6 mice

Supplementary Table 1. Clinical data

Code	Age	Gender	WBC (10K/µL)	Cytogenetics	Molecular data	KS99 IC50 (nM)
1341	83	M	201	46,XY,t(1;3)(p34.1;q27), t(2;18)(q31;q11.2), del (11)(p11.2p15)	CBL, KIT	439.9
1290	74	M	99.07	46,XY	ASXL1, DNMT3A, IDH1, KRAS, NRAS, RUNX1	444.1
1103	41	F	247.25	46,XX	FLT3-ITD, NOTCH1, PTPN11	505.3
1356	64	M	72.7	46,XY,inv(16)(p13.1q22)[19]/49,idem,+6,+8,+22[1]	KIT	507.4
1273	72	F	13.4	46,XX	ASXL1, CEBPA, NRAS, SRSF2, TET2 (95%)	539.6
1316	40	M	41.1	46,XY	NPM1, IDH1, TET2, KRAS, NRAS, PTPN11	619.5
1364	76	M	168	46,XY	NPM1, FLT3-ITD, DNMT3A	764.7
1265	74	M	217.36	46,XY,t(7;11)(p15;p15)	FLT3-ITD, HOXA9/NUP98 FUSION	818.1
990	69	M	106.66	46,XY,del(13)(q12q14)[2]/46,XY[18]	U2AF1	827.6
1360	59	F	10,4	46,XX	FLT3-ITD, ASXL1, WT1	836.7
1278	80	M	40.25	46,XY	ASXL1, CBL, RUNX1, EZH2	947.5
1172	46	F	149.23	46,XX	FLT3-ITD	956.9
1292	72	M	78.31	46,XY,t(7;11)(p15;p15)	TET2	1125
1071	81	M	148.89	47,XY,+8[18]	N/A	1357
1321	40	F	71.31	46,XX	FLT3-ITD, DNMT3A, TET2	1375
1241	50	F	180.4	46,XX	NPM1, FLT3-ITD	1381
1256	50	F	65.45	46,XX	NPM1, FLT3-ITD	1383
1257	45	M	160.32	46,XY	NRAS, IDH1, NPM1, and PTPN11	1408
934	72	F	137.78	46,XX	NPM1, FLT3-ITD	1439
1340	80	F	42.73	46,XX	NPM1, DNMT3A, NRAS	1474
1081	58	M	289.74	46,XY	NPM1, FLT3-ITD	2990
1099	86	M	141.02	46,XY	NPM1	-

Supplementary Table 2. Autodock analysis complied

Complex name	Binding energy (Kcal/mol)	Ligand energy (Kcal/mol)	Intermolecular energy (Kcal/mol)	Inhibition constant	Ligand binding pocket	H-bond
ALDH1A1-KS99	-9.65	-0.4	-10.84	84.19 nM	TRP178,MET175, ASN170,PHE466, PHE171,CYS303, VAL460,TYR297, SER121, CYS302, ILE304, GLY294, GLY458,TYR457, HIS293, PHE290	ASN170
STAT3-KS99	-6.76	-0.28	-7.95	11.14 uM	ALA250, ILE258, CYS251,GLN326, PRO336,PRO333, ASP334, SER514, GLY254,ARG335, TRP474, GLY253, PRO256, PRO255, TRP510, SER513	CYS251 SER514
STAT3-KS99 Phospho-tyrosine binding site	-6.97	-0.29	-8.16	7.8 uM	CYS712, PRO715, VAL713, THR714, TYR640, TYR657, GLU638, VAL637, TRP623, SER636. GLN635	TYR640 TYR657 GLU638