

Pharmacological blockade of rho kinase enhances venetoclax responses in translational models of acute myeloid leukemia

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Supplementary Appendix:

Title: Pharmacological blockade of rho kinase enhances venetoclax responses in translational models of acute myeloid leukemia

Running Title: Targeting ROCK improves venetoclax response in AML

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Materials and Methods:

Materials

DJ4 was synthesized at the Organic Synthesis Core (RRID:SCR_012425) of the Penn State College of Medicine, as reported previously.¹ Fasudil (Cat# A702414), GSK269962A (Cat# A266066), and venetoclax (Cat# A156386) were procured from AmBeed (Buffalo Grove, IL, USA). The cytokines stem cell factor (SCF; #100-04), FLT3 ligand (FLT3-L; #100-21AF), and GM-CSF (#100-08) were purchased from FUJIFILM Irvine Scientific (Santa Ana, CA). Interleukin-3 (IL-3; #203-IL) was obtained from R&D Systems (Minneapolis, MN), and granulocyte colony-stimulating factor (G-CSF; Neupogen®) was obtained from the pharmacy. StemRegenin1 (SR1; # S2858) was purchased from Selleckchem (Houston, TX). Lentiviral vector pLV-EF1a-LUC2-T2A-EGFP was procured from VectorBuilder (Chicago, IL, USA). All other reagents used were of molecular biology grade.

Clinical samples

Bone marrow aspirates or peripheral blood samples were obtained from AML patients during diagnosis or disease relapse after informed consent using protocols approved by the Institutional Review Board (IRB# 2000-186) of the Penn State College of Medicine. Cord blood (CB) samples were obtained from freshly delivered placentas of healthy donors at Penn State Health Obstetrics and Gynecology. Mononuclear cells (MNCs) were isolated through density gradient separation (Ficol-Paque, GE Healthcare Life Sciences, Pittsburgh, PA).

Cell culture

AML cell lines were cultured in either RPMI-1640 (#10-040; for MOLM-13, OCI-AML2, OCI-AML3, and U937) or IMDM (#10-016; for MV4-11, MV4-11/VENR, HL-60, and KG-1) growth media (Corning, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ThermoFisher Scientific, USA; #A5209502) and 1X Penicillin-Streptomycin (GIBCO, Gaithersburg, MD) at 37°C in a humidified incubator with 5% CO₂. AML cell lines HL-60 (#CCL-240, CVCL_0002), KG-1 (#CCL-246, RRID:CVCL_0374), and MV4-11 (#CRL-9591, RRID:CVCL_0064) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). VEN-resistant MV4-11 cells (MV4-11/VENR) were generated via continued subculture in increasing doses of VEN, beginning at 2nM and doubling until reaching a final concentration of 1 μ M as described earlier.² MV4-11/VENR cells were continually maintained in growth media with 1 μ M of VEN. MV4-11/VENR cells were transduced with pFU-LUC2-EGFP lentiviral vector to stably express GFP and luciferase as described previously.³ MV4-11 cells expressing YFP and luciferase were kindly provided by Kenichiro Doi.⁴ U937 cells stably expressing the firefly

luciferase (LUC2) and tdTomato (Addgene plasmid #72486, RRID: Addgene_72486) were kindly gifted by Kazuhiro Oka. Other AML cell lines, including OCI-AML2, OCI-AML3, MOLM-13, MOLM-13-YFP-Luc, and U937, were obtained as mentioned in the acknowledgments.

Primary human AML cells were cultured in the serum-free medium StemSpan SFEM II (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with cytokines (SCF, IL-3, FLT3-L, G-CSF, GM-CSF), StemRegenin1 (SR1), and 1X Penicillin-Streptomycin at 37°C in a humidified incubator with 5% CO₂ as described earlier.⁵ AML patient samples were chosen based on the availability of adequate samples at the time of assay was performed.

Cell viability assays

The AML cell lines were seeded at 40,000 cells/well in 0.2 mL of media in a 96-well flat-bottom plate and treated with increasing concentrations of ROCK inhibitors (0.01-5 µM of DJ4, 5-125 µM of fasudil, 0.05-10 µM of GSK269962A-GSK) and VEN (0.01-20 µM) in combination. The cell viability was assessed by MTS assay after 24 h (VEN + DJ4 or fasudil) or 48 h (VEN + GSK) of treatment using MTS (CellTiter 96 Aqueous MTS reagent powder, #G1111, Promega) and phenazine methosulfate (PMS; #P9625, Sigma Aldrich) solution mix according to the manufacturer's protocol and as described earlier.^{6,7} Absorbance measured at 490 nm by Molecular Devices SpectraMax i3x Multi-Mode Microplate Reader (RRID:SCR_026346) was presented as % viability relative to vehicle control (DMSO).

The viability of primary AML patient cells after treatment with GSK (0.125-1 µM) and VEN (0.06-2 µM) alone or in combination was measured using CellTiter-Glo luminescence assay (Promega, Cat# G755B). In brief, primary AML patient cells 24-48h post-thaw were seeded in serum-free SFEM media supplemented with cytokines at a density of 3 x 10⁵ cells/mL in 96-well flat-bottom white plate to a final volume of 120 µL. After 48 h of treatment with increasing concentrations of GSK (0.125-1 µM) and VEN (0.06-2 µM) in combination, the cells were subjected to viability measurement using CellTiter-Glo reagent as per the manufacturer's protocol. Luminescence was measured using Molecular Devices SpectraMax i3x Multi-Mode Microplate Reader (RRID:SCR_026346) and represented as % viability relative to vehicle control. Viability data from drug combinations were used to generate dose-response matrices for synergy analysis.

Drug synergy analysis

Dose-response matrices containing % viabilities were used to compute synergy scores. The synergy between two drugs was analyzed using the SynergyFinder 2.0 web-application tool (<https://synergyfinder.fimm.fi/>, RRID:SCR_026127).⁸ Bliss reference model was used to quantify the degree of interaction (synergy or antagonism) between drugs. Default parameters were used (Readout = Viability; Detect Outliers = Yes; Curve Fitting = LL4). Bliss scores (corrected for baseline) greater than 10, between 10 and -10, and below -10 are considered synergistic, additive, and antagonistic, respectively.

Clonogenic assay

Viable cryopreserved primary AML patient samples and cord blood mononuclear cells (CB-MNCs) were thawed at 37°C in IMDM medium containing 10% FBS. The colony-forming ability of primary AML patient cells in the presence of either GSK (0.25-2.5 μ M) or VEN (0.5 μ M) alone or in combination was tested by seeding cells at equal density in Human Methylcellulose Complete Media (R&D Systems, Minneapolis, MN, USA) in a 12-well cell culture plate in triplicate and incubating for 10-14 days, as reported earlier.⁵ Colonies (>20 cells/colony) were counted under an Olympus CKX31 inverted light microscope (Olympus Corporation, Center Valley, PA, USA) and imaged using a 4x objective. The data was represented as % colonies relative to the solvent (DMSO) control. Samples were chosen based on the availability of adequate samples at the time of assay was performed.

Measurement of apoptosis, ROS, and mitochondrial membrane potential

The AML cells were seeded at a density of 4×10^5 cells/ml in a 12-well plate and left untreated or treated with vehicle (DMSO), GSK, VEN alone, or in combination at the indicated concentration. After 48 h of treatment, Annexin V positivity, Caspase-3/7 activity, reactive oxygen species (ROS) levels, and mitochondrial membrane potential ($\Delta\Psi_m$) were assessed using Muse Annexin V & Dead Cell Kit (Cytex, Cat# MCH100105), Muse Caspase-3/7 Kit (Cat# MCH100108), Muse Oxidative Stress Kit (Cat# MCH100111), and Muse MitoPotential Kit (Cytex, #MCH100110) respectively, following the manufacturer's instructions.^{5,7} For each sample, at least 2000 events were acquired using Muse Cell Analyzer (RRID:SCR_020252) and represented as a percentage of positive cells.

Assessment of mitochondrial membrane potential ($\Delta\Psi_m$) in whole intact cells

MV4-11 (naïve and VENR) cells were seeded at 0.4×10^6 cells/ml in IMDM growth media and treated with vehicle, indicated doses of either GSK, VEN, or the GSK+VEN combination. After 15 and 24 h of treatment, 3 ml of cells from each treatment were divided into unstained, vehicle, and FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) tubes with 1 ml each. After this, cells were treated with either vehicle (DMSO) or FCCP (5 μ M; Sigma, #C2920) for 15 min at 37°C. The cells were stained with 20 nM of Tetramethylrhodamine methyl ester (TMRM; Thermo Fisher Scientific, #T668) and incubated for 30 min at 37°C. Thereafter, 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, #62248) was added at 1 μ g/ml. Samples were then analyzed using BD LSRFortessa flow cytometer (BD Bioscience) at the core facility of Pennsylvania State University. Data was analyzed by excluding nonviable cells (DAPI positive), and the median fluorescence intensity of TMRM normalized to mitochondrial uncoupler FCCP (negative control) was expressed as % of the vehicle group.² FlowJo software version 10.0.7 (RRID:SCR_008520) was used for data analysis.

Mitochondrial respiratory profiling in intact and permeabilized cells

High-resolution respirometry measurements were performed at 37°C using the Oroboros Oxgraph-2k (O2k; Oroboros Instruments, Innsbruck, Austria) in a 0.5 - 1 mL reaction volume and normalized to viable (trypan blue exclusions) cell count as described earlier.² For respiratory flux (OXPHOS) measurements in intact cells, MV4-11/VENR cells were resuspended in Intact Cell Respiratory Media (17.7 g/L Iscove's Modified Dulbecco's Medium (IMDM), 20 mM HEPES, 1% Penicillin/Streptomycin, 10% FBS, pH 7.4) after overnight treatment either with vehicle or 400 nM GSK. After basal respiration was established, the maximal rate of respiration was induced by FCCP titration (0.5–5 mM). For permeabilized cell measurements, MV4-11/VENR cells were resuspended in Respiratory Buffer supplemented with creatine (105 mM MES potassium salt, 30 mM KCl, 8 mM NaCl, 1 mM EGTA, 10 mM KH_2PO_4 , 5 mM MgCl_2 , 0.25% BSA, 5 mM creatine monohydrate, pH 7.2) after overnight treatment with either vehicle or 400 nM GSK. Cells were permeabilized using 0.02 mg/mL of digitonin, and then the respiratory capacity of different complexes was measured by energizing the mitochondria with inhibitors and carbon substrates as follows: pyruvate, malate, and ADP (Complex I; 5/1/2 mM), rotenone (Rot, 0.5 mM), succinate (Complex II; 5 mM), malate (Malo; 10 mM), and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD)/ascorbate (Asc) (Complex IV; 0.05/2 mM).

Western blotting

The AML cell lines (MV4-11 and MV4-11/VENR) were seeded at a density of 4×10^5 cells/ml in T25 or T75 cell culture flasks and left untreated or treated with DMSO (vehicle), GSK, VEN alone, or in combination at the indicated doses and time. After the incubation period, the cells were pelleted, and whole cell lysates were prepared in RIPA buffer (Sigma; #R0278) supplemented with 1X Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFisher Scientific; #78442) prior to being subjected to immunoblotting analysis as described earlier.⁶ Briefly, equal amounts of protein after BCA assay quantification were subjected to gel electrophoresis using NuPAGE 4–12% Bis-Tris gel (Life Technologies, Carlsbad, CA, USA). Proteins were transferred to PVDF membrane prior to probing with the following antibodies: ROCK1 (BD Biosciences; #611136, RRID:AB_398447), ROCK2 (BD Biosciences; #610623, RRID:AB_397955), Cofilin (Cell Signaling; #5175S, RRID:AB_10622000), phospho-Cofilin (Cell Signaling; #3313S, RRID:AB_2080597), Drp1 (Cell Signaling; #5391, RRID:AB_11178938), phospho-Drp1 (Cell Signaling; #4494, RRID:AB_11178659), phospho-PTEN (Cell signaling; #9549S, RRID:AB_659891), NOXA (Santa Cruz Biotechnology; #sc-56169, RRID:AB_784877), Bcl-2 (Cell signaling; #3498S, RRID:AB_1903907), phospho-Bcl-2 (Cell signaling; #2827S, RRID:AB_659950), Mcl1 (Cell signaling; #5453, RRID:AB_10694494), Bcl-xL (Cell signaling; #2764S, RRID:AB_2228008), Bak (Cell signaling; #12105S, RRID:AB_2716685), PARP (Cell signaling; #9542S, RRID:AB_2160739), Caspase-3 (Cell signaling; #9662S, RRID:AB_331439), β -Actin (Santa Cruz Biotechnology; #sc-47778, RRID:AB_626632), and GAPDH (Santa Cruz Biotechnology; #sc-32233, RRID:AB_627679). The goat anti-rabbit IgG–horseradish peroxidase (HRP) conjugates (#7074S, RRID:AB_2099233) and the horse anti-mouse IgG-HRP conjugates (#7076S, RRID:AB_330924) were purchased from Cell Signaling Technology. The chemiluminescent signals were captured using the Bio-Rad ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA, USA) and quantified via the ImageJ software (<https://imagej.net/ij/>, RRID:SCR_003070). The band densities were relative to GAPDH levels and normalized to an untreated reference that was set to 1.

In vivo mice efficacy and safety studies

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), and the mice were procured either from Jackson Lab, Charles River Laboratory, or bred at Penn State University.

Cell line-derived xenografts (CDX): Cell line-derived xenografts were established to test *in vivo* efficacy of GSK and VEN combination as described previously.⁵⁻⁷ Briefly, luciferase-expressing AML cell lines (2.5×10^6 of

MV4-11-YFP-Luc, 2×10^6 of MV4-11/VENR-EGFP-Luc, 0.5×10^6 of MOLM-13-YFP-Luc, and 1×10^4 of U937-Luc- tdTomato) were intravenously injected into 6-9-week-old NOD Cg-Rag1tm1Mom Il2rgtm1Wjl Tg(CMV-IL3,CSF2,KITLG)1Eav/J (NRG-S) mice (RRID:IMSR_JAX:024099) and randomized to experimental groups based on bioluminescence imaging (BLI) signals and body weight. All female or male mice were used per study, and the mice were treated with either vehicle, GSK (10mg/kg, i.p., Mon-Fri), VEN (50-100 mg/kg, p.o., Mon-Fri), or both GSK and VEN for 2-3 weeks as indicated. VEN was formulated in 10% ethanol/30% polyethylene glycol 400 (PEG400)/60% phosal 50 propylene glycol (PG) (v/v/v),⁹ and GSK was dissolved in 20% PEG300/0.25% Tween-80/79.75% water,¹⁰ as reported earlier. Dosing regimens were selected based on published pharmacokinetic data for VEN and GSK269962A. VEN has been reported to exhibit oral half-lives of approximately 4–6 h in mice and 6–10 h in rats, with systemic exposures sufficient to support once-daily dosing.^{11,12} In contrast, GSK269962A demonstrates a shorter reported half-life of ~1–3 h with moderate oral bioavailability in rodents,¹³ supporting the dosing schedules used in this study. The mice were regularly imaged once a week or as indicated using the Perkin Elmer IVIS Lumina LT Series III imaging system (RRID:SCR_018621) after 7 min of D-luciferin (Gold Biotechnology, St. Louis, MO) intraperitoneal injection (150 mg/kg). The leukemia burden was quantified using Living Image software (Perkin Elmer) and represented as average radiance (p/s/cm²/sr). After the treatment period, peripheral blood samples were collected through submandibular puncture and subjected to flow cytometry analysis. The mice were either monitored for overall survival and subjected to Kaplan–Meier analysis or bone marrow (BM) cells were harvested to evaluate leukemic burden by flow cytometry.

Patient-derived xenograft (PDX): PDX model was first established by injecting AML Pt. 1265 primary cells (24h post-transduction with pLV-EF1a-LUC2-T2A-EGFP lentiviral vector) into NRG-S mice as described earlier with modifications.^{14,15} Briefly, the primary AML cells were mixed with anti-human CD3 (OKT-3) monoclonal antibody (Bio X Cell, #BE0001-2, RRID: AB_1107632; for T-cell depletion *in vivo*) prior to injection into NRG-S mice irradiated with 200 rad of γ -rays a day prior. The BM cells collected from PDX mice were sorted for GFP positivity, cultured in serum-free growth medium, and injected into secondary recipient mice for enrichment of labeled patient cells. The BM cells collected from secondary PDX mice were transplanted into NRG-S mice for establishing the bioluminescent PDX model. After 13 days of transplant, the mice were randomized into four experimental groups (control, GSK, VEN, and GSK+VEN) based on BLI signals (n=5-7). The mice were treated

with either GSK (30 mg/kg, p.o.), VEN (50 mg/kg, p.o.), or the combination of GSK and VEN once daily for 6 days/week for a total of 3 weeks. After the end of the treatment, the leukemia burden was periodically monitored by IVIS imaging. On day 148 (21 weeks) post-transplant, the bone marrow cells were collected from all the experimental groups and checked for human AML (donor) chimerism by flow cytometry.

Mouse safety study: The short (2 weeks) and long-term subchronic (4 weeks) safety and toxicity of the GSK269962A and VEN combination were evaluated by treating 6- to 8-week-old female Swiss Webster mice (Charles River Laboratory, RRID:IMSR_CRL:024) with either vehicle, GSK (30 mg/kg, p.o.), VEN (50 or 100 mg/kg, p.o.), or the GSK+VEN combination once daily, six days per week (Mon-Sat), for two or four weeks. After 2- or 4-weeks of treatment, blood samples were collected from mice through cardiac puncture to run a complete blood count (CBC) by Hemavet 950FS analyzer (Drew Scientific, Plantation, FL, USA) and clinical chemistry for assessing the renal and liver functions (VRL Diagnostics, Gaithersburg, MD, USA).

Flow cytometry

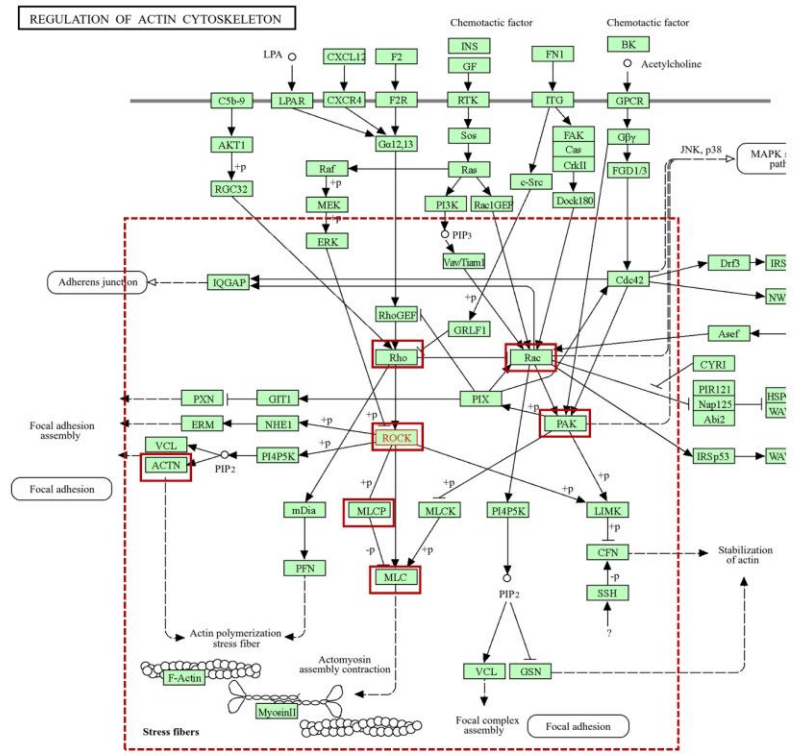
The peripheral blood or bone marrow cells collected from CDX or PDX mice were analyzed for the presence of human AML cells (CD45 positive) by flow cytometry.^{6,16} Briefly, whole blood or bone marrow cells were treated with BD Pharm Lyse Lysing Buffer (BD Biosciences, #555899) to lyse red blood cells followed by washing with fluorescence-activated cell sorting (FACS) buffer (Dulbecco's phosphate buffered saline with 2% FBS). Cells were incubated with cocktail of Human BD Fc Block (BD Biosciences, #564220, RRID:AB_2869554) and Mouse BD Fc Block (BD Biosciences, #553141, RRID:AB_394656) for 10 minutes at room temperature to prevent nonspecific antibody binding and then stained with following antibodies: human CD45-APC/Cy7 (BioLegend, #304014, RRID:AB_314402) or human CD45-PE (BioLegend, #368510, RRID:AB_2566370), human CD34-FITC (BD Biosciences, #555821, RRID:AB_396150), mouse CD45-BV650 (BD Biosciences, #563410, RRID:AB_2738189) cocktail for 20 minutes on ice in the dark, followed by washing with FACS buffer. Finally, the cells were suspended in FACS buffer with viability dye 7-AAD (BioLegend, #420404) and analyzed by BD LSR Fortessa Flow Cytometer (BD Bioscience) and processed using FlowJo version 10.0 (RRID:SCR_008520). Engraftment of human cells (chimerism) in peripheral blood or bone marrow was detected using the appropriate fluorochrome filters and reported as the percentage of positive cells.

Supplementary Figures:

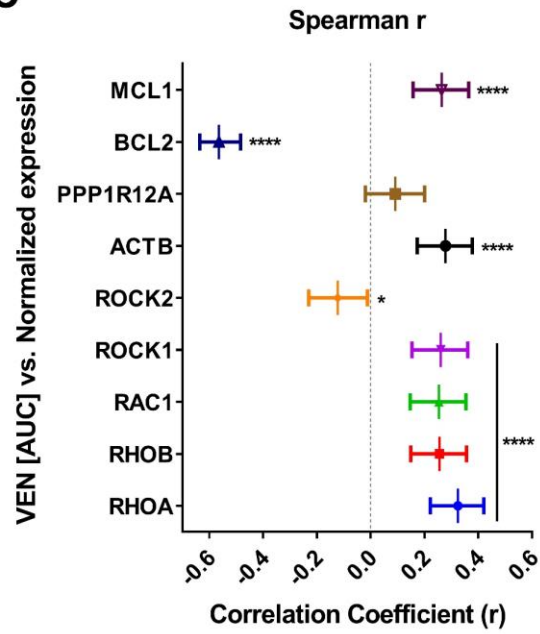
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		Day 16	
S.No.	Target Gene	delta CS	p value
1	RHOA	-3.657	0.0505
2	ROCK1	-0.410	0.7850
3	ROCK2	-1.488	0.3017
4	MYL2	-2.364	0.0347
5	MYL9	-2.388	0.1719
6	PPP1CA	-1.243	0.1569
7	PPP1CB	-0.645	0.3618
8	PPP1CC	-1.450	0.0195
9	PPP1R12A	-2.224	0.0116
10	PPP1R12B	-1.847	0.1616
11	PIP5K1A	-3.130	0.0058
12	PIP5K1C	-2.211	0.1457
13	RAC1	-0.314	0.8853
14	RAC2	-2.358	0.0185
15	RAC3	-1.410	0.1617
16	PAK1	-2.001	0.0915
17	PAK2	-0.978	0.3542
18	PAK3	-2.460	0.0030
19	PAK4	-2.228	0.0233

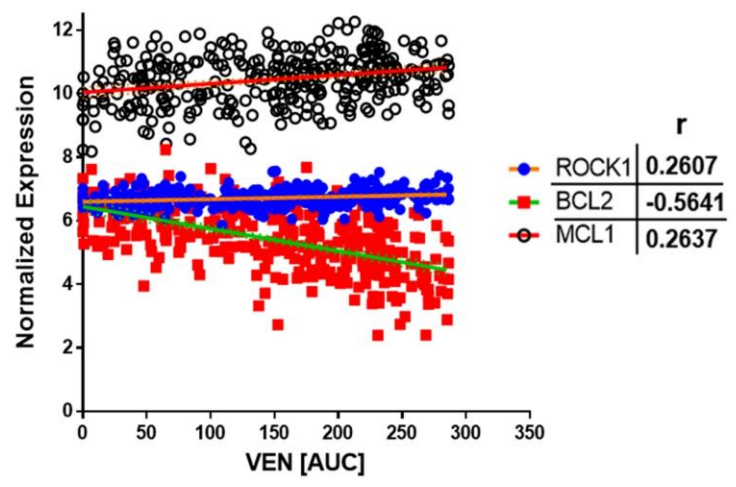
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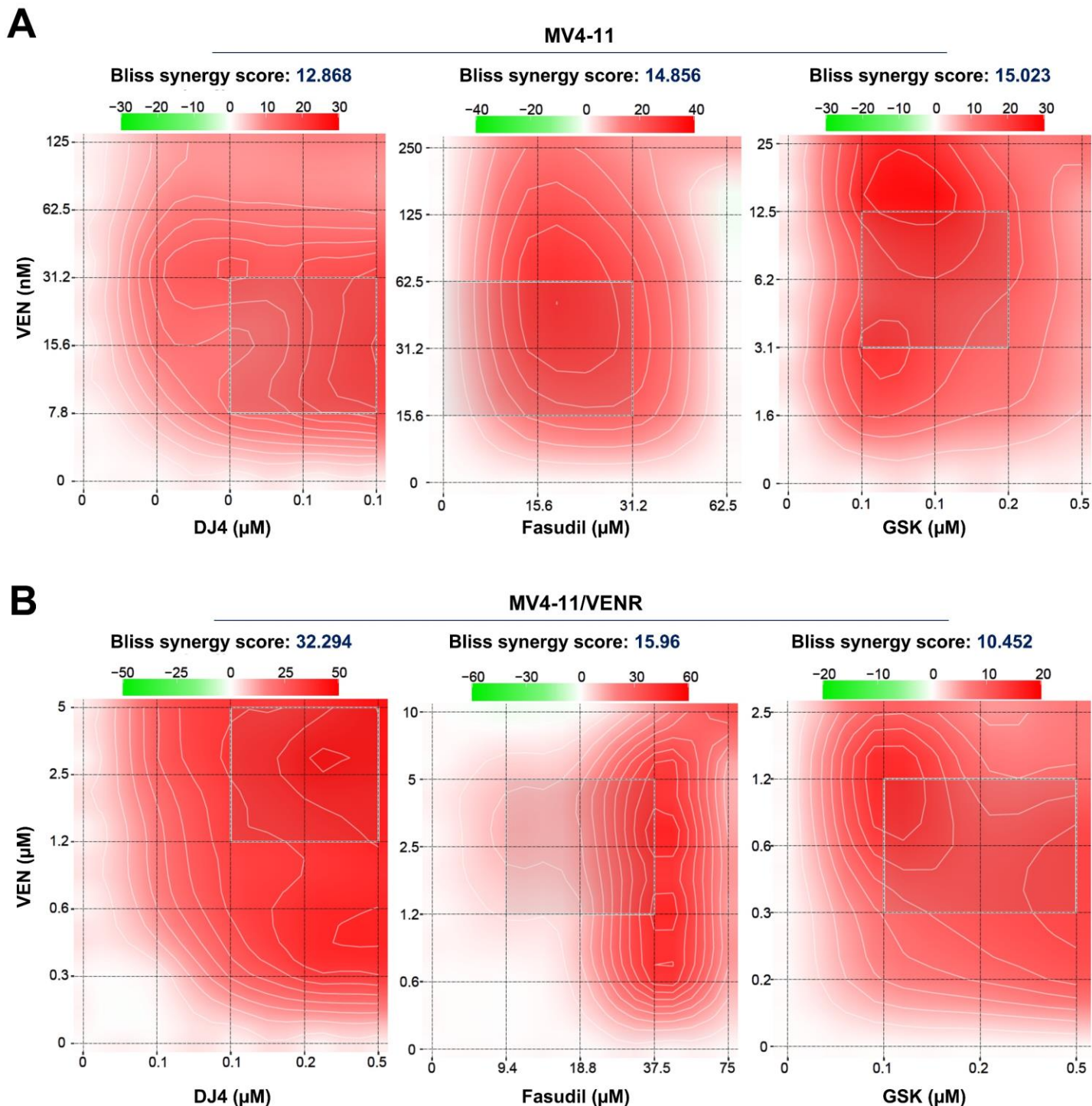
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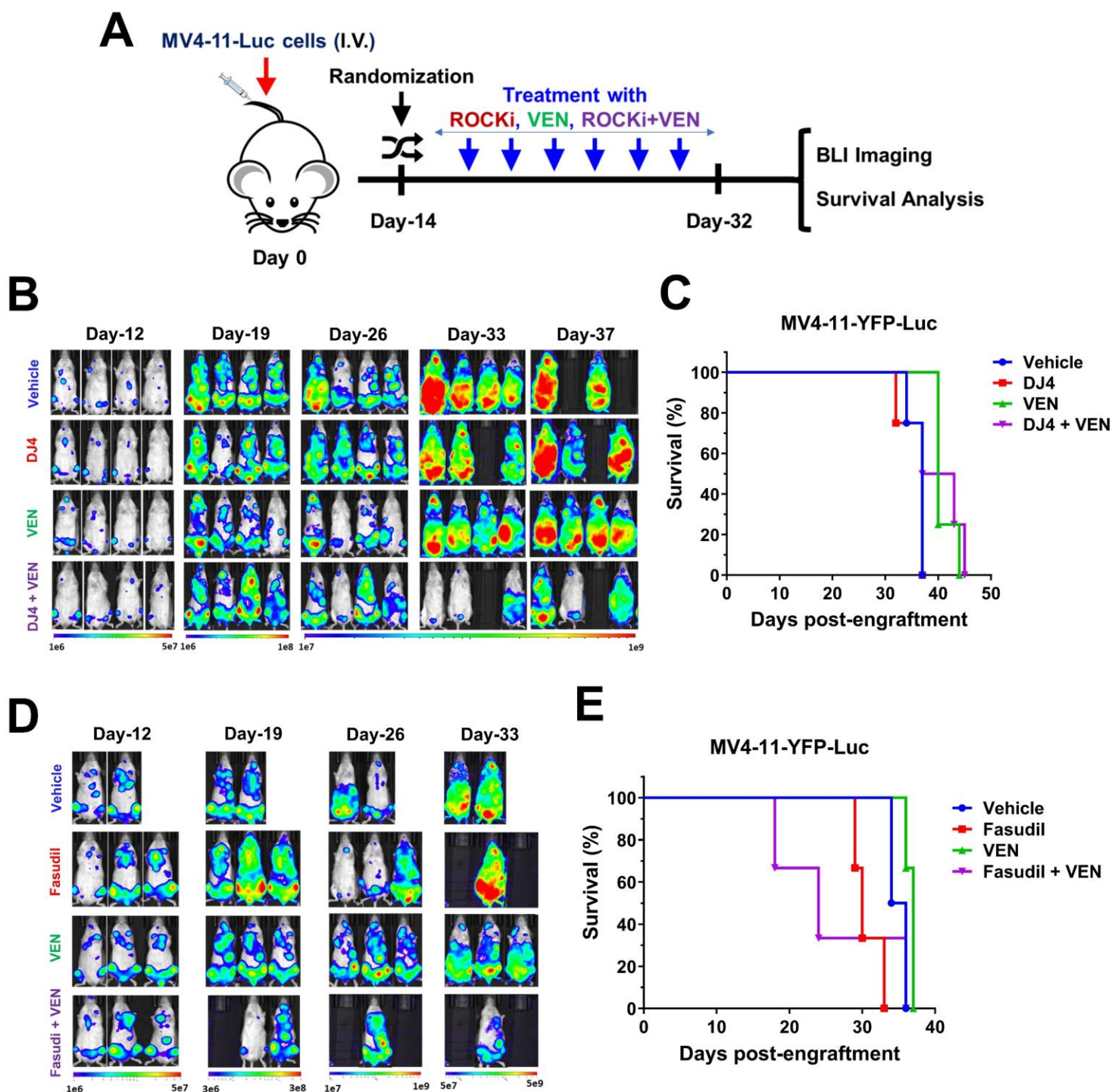
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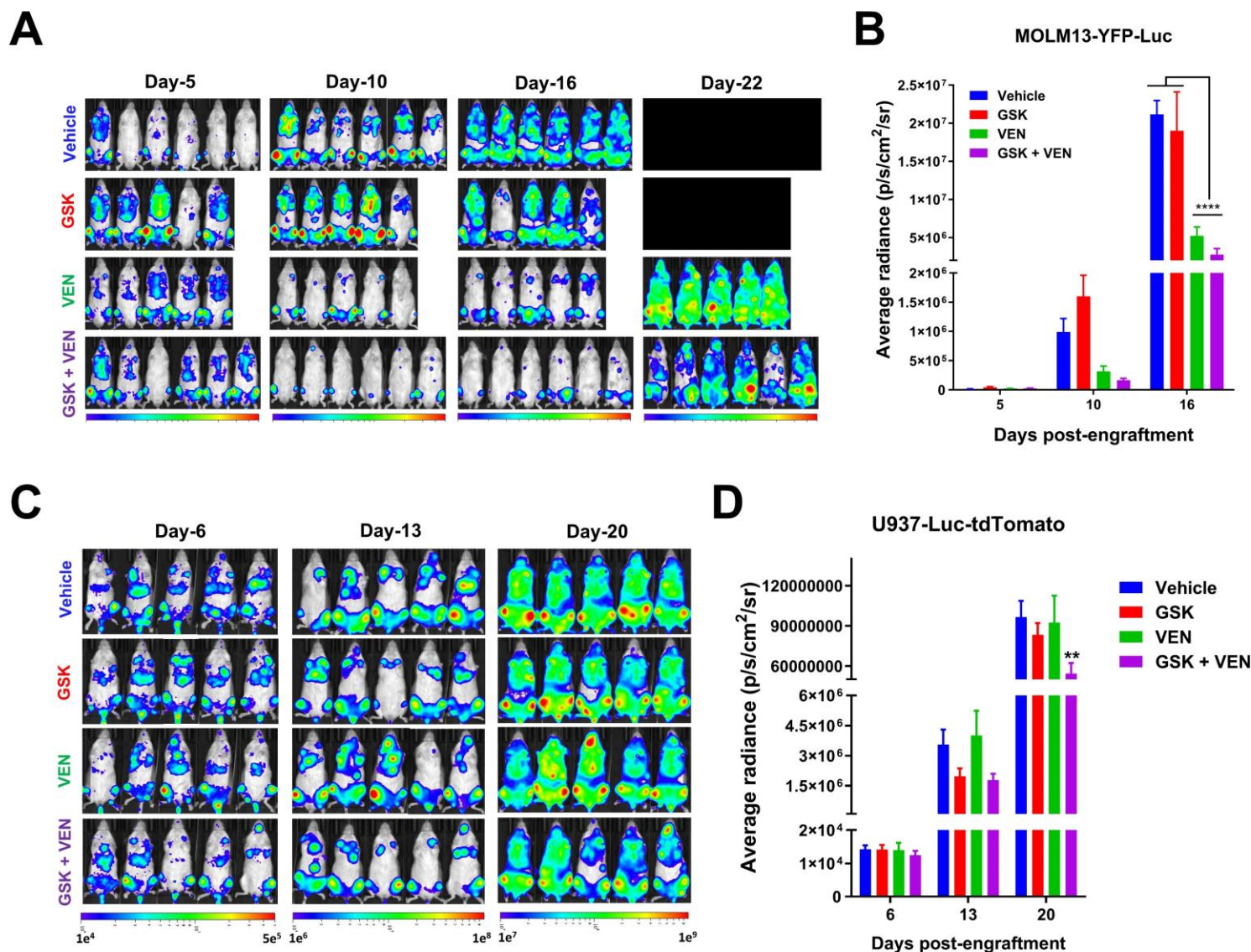
Supplementary Figure 1: The Rho/ROCK pathway cooperates with venetoclax activity in AML cells. A) Rho/ROCK pathway genes are negatively enriched (genes whose loss synergizes with venetoclax) in a CRISPR/Cas9 loss-of-function screen in MOLM-13 cells after 16 days of VEN treatment. The delta CRISPR scores (CS) and the corresponding p-values for the selected genes were adopted from Chen X et al. ¹⁷. B) KEGG pathway map (<https://www.kegg.jp/entry/map04810>) for 'Regulation of actin cytoskeleton' highlights the Rho/ROCK pathway genes that are negatively enriched in the CRISPR loss-of-function screen after VEN treatment. C & D) Spearman correlation analysis comparing venetoclax area under the curve (AUC) against normalized mRNA expression of BCL-2, MCL1, and Rho/ROCK pathway genes. The correlation coefficient (r) for different genes (C) and a representative correlation plot for BCL-2, MCL1 and ROCK1 genes (D) was shown. We used MCL1 and BCL-2 genes as controls, and accordingly, their expression levels resulted in a positive and negative correlation with VEN AUC, respectively. Data (n=332) were obtained from BeatAML2.0 (<http://www.vizome.org/aml2/>). *p<0.05 and ****p<0.0001 (two-tailed) indicate statistical significance.



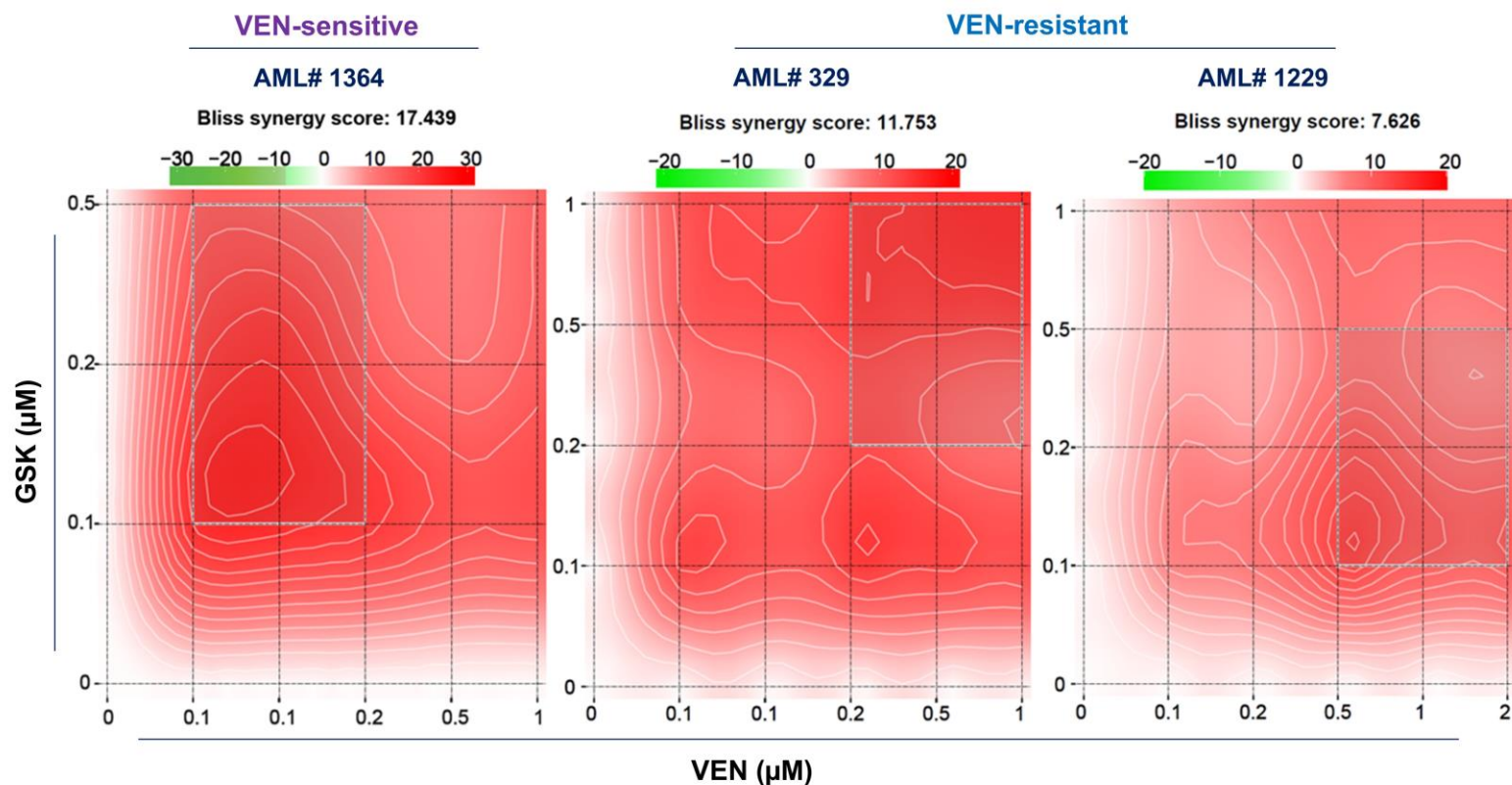
Supplementary Figure 2: ROCK inhibitors exhibit synergistic cytotoxicity with venetoclax combination in AML cell lines *in vitro*. A & B) Heatmap showing the Bliss synergy score in MV4-11 (A) and MV4-11/VENR (B) cells after ROCKi and VEN combination treatment for 24h (DJ4, Fasudil) and 48h (GSK269962A-GSK). Bliss scores greater than 10 were considered synergistic. Representative plots from at least three independent experiments are presented.



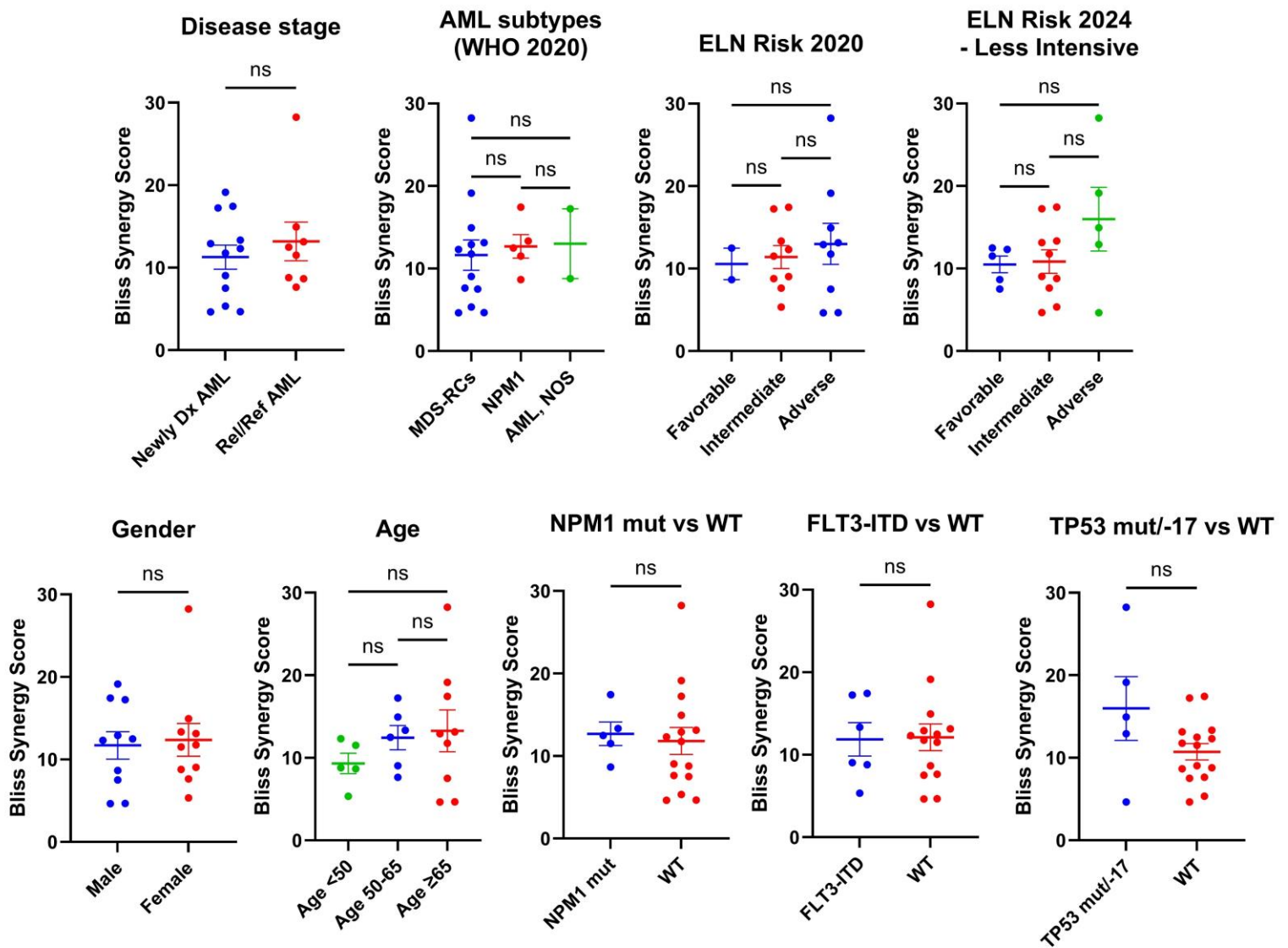
Supplementary Figure 3: Combination of VEN with ROCKi (Fasudil and DJ4) exhibit modest efficacy in the MV4-11 CDX mouse model. A) Schematic depicting the randomization of NRG-S (n=2-4) mice into four experimental groups (vehicle, ROCKi-DJ4 or fasudil, VEN, and ROCKi+VEN combo) based on BLI signals after 12 days of post-injection of MV4-11-YFP-Luc cells intravenously. Fasudil (25mg/kg; i.p.; M-F) or DJ4 (10mg/kg; i.p.; M-F) combined with VEN (100mg/kg; p.o.; M-F) for mice treatments. B & D) The MV4-11 CDX mice were monitored regularly by IVIS imaging at indicated days. C & E) Kaplan-Meier survival analysis of MV4-11 CDX mice after treatment with ROCKi (DJ4 or Fasudil), VEN, and the ROCKi+VEN combination. The Gehan-Breslow-Wilcoxon test indicated that the overall survival of mice was not statistically different for treatment groups compared to vehicle control.



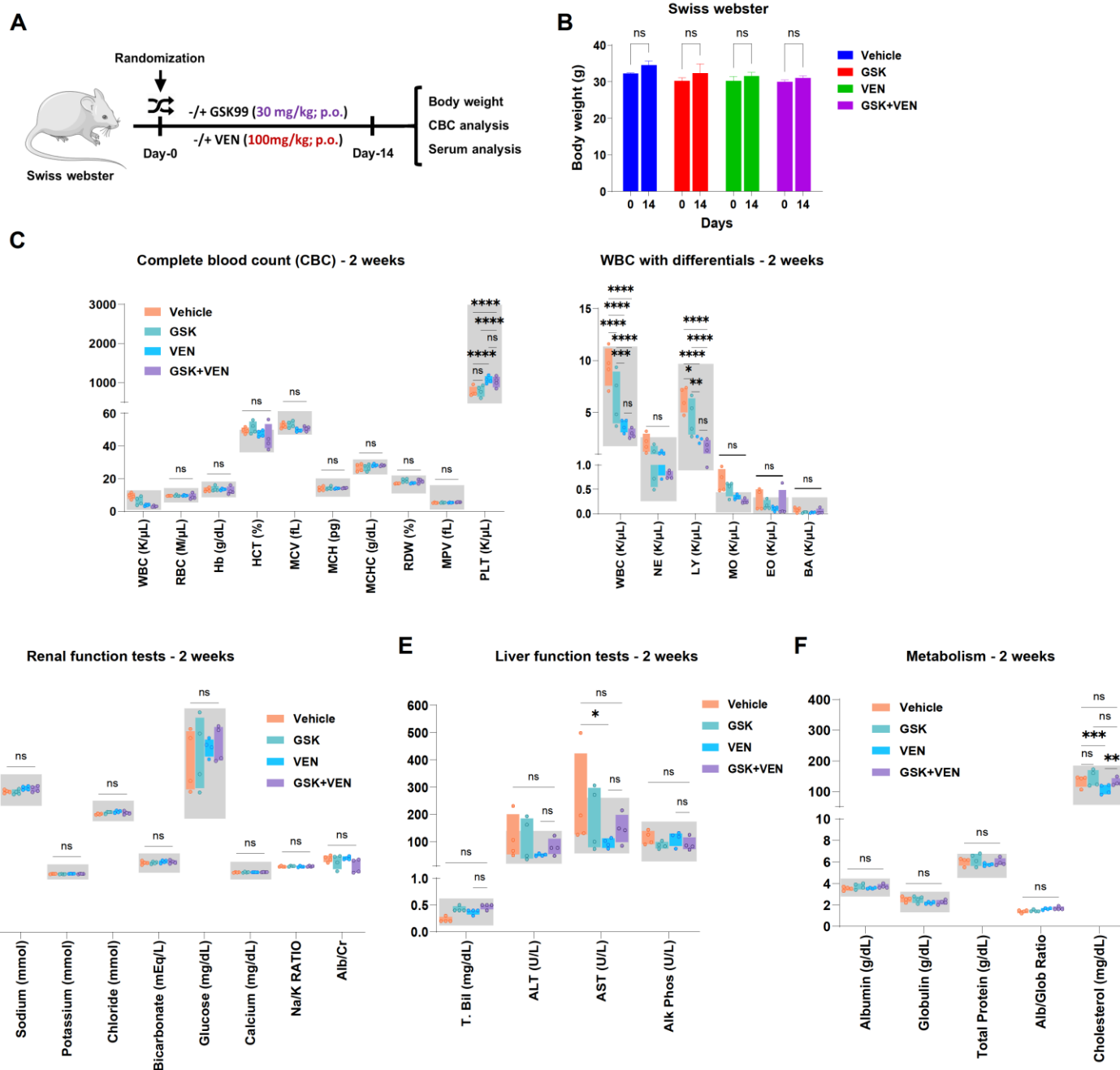
Supplementary Figure 4: GSK and VEN combination synergistically decrease leukemia burden in AML cell line xenograft models. A & C) Bioluminescence imaging of MOLM13-YFP-Luc (A) and U937-Luc-tdTomato (C) CDX mice at indicated days, as described in Figure 2. The NRG-S mice (n=5-6) were intravenously injected with AML cells (MOLM13-YFP-Luc, and U937-Luc-tdTomato) and randomized into experimental groups (Vehicle, GSK, VEN, and GSK+VEN). B & D) The leukemia burden in the MOLM13-YFP-Luc (B) and U937-Luc-tdTomato (D) CDX mice was quantified in the terms of BLI signals and presented as average radiance (p/s/cm²/sr). **p<0.01, ****p<0.0001 by 2-way ANOVA (Tukey's multiple comparisons test) compared to vehicle control.

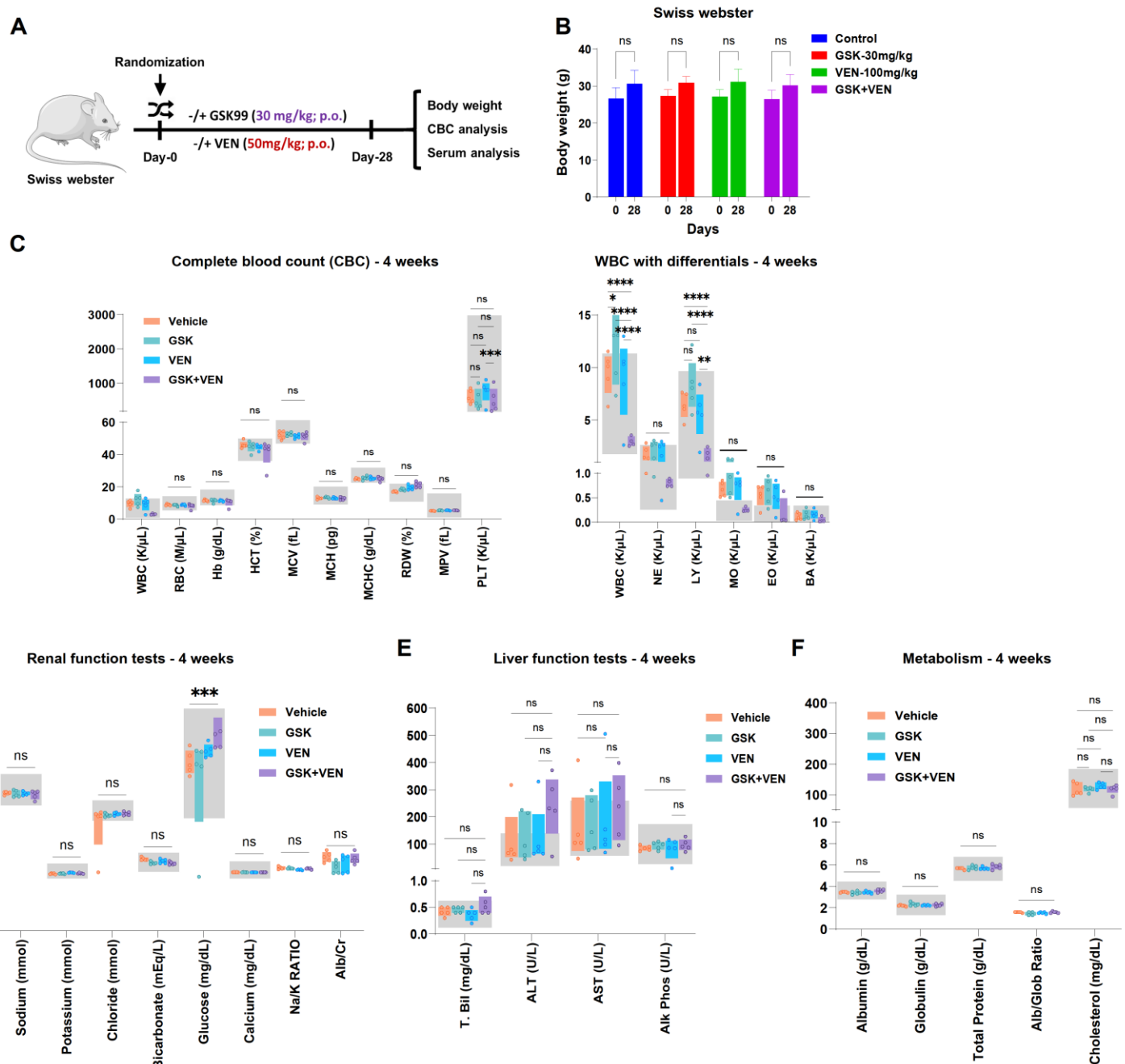


Supplementary Figure 5: ROCK inhibitors exhibit synergistic cytotoxicity with venetoclax combination in primary AML patient cells *ex vivo*. Heatmap showing the Bliss synergy score for primary AML patient cells (VEN-sensitive and VEN-resistant) after GSK and VEN combination treatment. The cell viability of patient cells was measured by the CellTiter-Glo assay. Bliss scores greater than 10 indicate 'synergy,' and 0-10 indicate 'additive' activity.



Supplementary Figure 6: Subgroup analysis of Bliss synergy scores for primary AML patient cells after treatment with GSK and VEN combination. Comparative analyses of the Bliss synergy scores after 48h of GSK+VEN combination treatment among subgroups of primary AML patient cells (n=20) disease characteristics, as defined according to baseline demographic and disease characteristics. Data are presented as mean $\pm$ SEM and * $p < 0.05$ is considered statistically significant when analyzed by either unpaired t-test (two groups) or one-way ANOVA (three groups). 'ns' denotes 'not significant'.





Supplementary Figure 8: Long-term (4-weeks) safety profile of GSK alone and in combination with VEN. A) Schematic depicting the randomization of Swiss Webster mice (n=5) into four experimental groups (vehicle, GSK, VEN, and GSK+VEN combo). B) Body weight (g) of experimental Swiss Webster mice before and after 4-weeks of treatment with either vehicle, GSK (30 mg/kg, p.o., Mon-Sat), VEN (50 mg/kg, p.o., Mon-Sat), or GSK+VEN combination. C) CBC analysis of normal Swiss Webster mice after 4-weeks of treatment. D-F) Serum collected from Swiss Webster mice after 4-weeks of treatment and subjected to clinical chemistry. The shaded area represents the normal ranges for each parameter. Data are presented as mean $\pm$ SD (n=5), and *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 were considered statistically significant compared to vehicle when analyzed by 2-way ANOVA (Tukey's multiple comparisons test). 'ns' denotes 'not significant'.

Supplementary Tables

Supplementary Table 1: IC₅₀ values for AML cell lines after 24h (VEN, DJ4, Fasudil) and 48h (GSK) of treatment assessed by MTS assay.

AML cell line	IC ₅₀ Concentration (μM)			
	VEN	DJ4	Fasudil	GSK
MV4-11	0.036	0.145	29.68	0.143
MV4-11/VENR	>5	0.257	42.25	0.387
MOLM-13	0.02	0.307	66.23	3.08
HL-60	0.033	1.097	53.97	>5
OCI-AML2	0.007	1.075	26.98	1.07
OCI-AML3	7.63	1.14	47.62	1.19
KG-1	>10	1.548	32.43	1.89
U937	>10	1.89	116	>5

Supplementary Table 2: Serum biochemistry analysis of renal, liver, and metabolic parameters in mice treated with vehicle, GSK, VEN, or the GSK+VEN combination for 2 or 4 weeks.

Renal function	Normal ranges	2 weeks				4 weeks			
		Vehicle	GSK	VEN	GSK+VEN	Vehicle	GSK	VEN	GSK+VEN
Creatinine (mg/dL)	0.12 - 0.43	0.11 ± 0.02	0.16 ± 0.11	0.1 ± 0.01	0.24 ± 0.14	0.1 ± 0.02	0.22 ± 0.12	0.2 ± 0.16	0.11 ± 0.03
Sodium (mmol)	124.81 - 160.31	151.48 ± 2.87	150.35 ± 4.37	157.55 ± 3.63	155.93 ± 5.29	150.16 ± 2.61	148.86 ± 5.97	149.62 ± 3.17	147.38 ± 7.69
Potassium (mmol)	2.17 - 8.18	7.44 ± 0.33	7.22 ± 0.17	7.85 ± 0.29	7.52 ± 0.29	8.26 ± 1.01	8.44 ± 0.83	10.03 ± 0.55	8.92 ± 0.9
Chloride (mmol)	99.96 - 121.75	112.1 ± 1.21	114.88 ± 1.92	116.23 ± 2.03	114.03 ± 2.88	91.98 ± 45.53	111.36 ± 3.11	113.48 ± 2.61	113.98 ± 3.41
Bicarbonate (mEq/L)	5.86 - 30.91	26.9 ± 3	26.95 ± 2	28.9 ± 2.59	28.38 ± 2.95	34.22 ± 2.76	27.44 ± 2.25	30.48 ± 3.94	26.64 ± 2.84
Calcium (mg/dL)	8.28 - 12.27	10.23 ± 0.5	10.45 ± 0.73	10.38 ± 0.13	10.65 ± 0.37	10.98 ± 0.39	10.84 ± 0.29	10.68 ± 0.15	10.88 ± 0.18
Na/K RATIO		20.5 ± 1	21 ± 0.82	20.25 ± 1.26	20.75 ± 0.96	18.6 ± 2.41	17.8 ± 1.3	15.2 ± 0.84	17 ± 1.22
Alb/Cr		34.23 ± 5.65	29.15 ± 12.43	34.84 ± 2.91	21.18 ± 12.28	37.8 ± 9.01	19.83 ± 10.41	26.64 ± 15.84	34.68 ± 9.61

Liver function	Normal ranges	2 weeks				4 weeks			
		Vehicle	GSK	VEN	GSK+VEN	Vehicle	GSK	VEN	GSK+VEN
T. Bil (mg/dL)	0.17 - 0.53	0.23 ± 0.05	0.43 ± 0.05	0.38 ± 0.05	0.48 ± 0.05	0.42 ± 0.08	0.46 ± 0.05	0.36 ± 0.11	0.54 ± 0.17
Alk Phos (U/L)	62-209	114.5 ± 25	86.75 ± 12.5	112 ± 25.97	90.75 ± 23.82	114.2 ± 114.7	127.8 ± 86.57	125.2 ± 114.98	236.8 ± 118.1
ALT (U/L)	28-132	113.25 ± 81.96	109.5 ± 79.71	51.25 ± 4.65	81 ± 30.56	160.2 ± 142.69	173.2 ± 101.22	189 ± 179.86	235.4 ± 123.71
AST (U/L)	59-247	237.75 ± 177.11	187.75 ± 118.25	94.75 ± 17.46	147.5 ± 53.21	85.4 ± 9.5	94.8 ± 14.6	81.2 ± 41.52	96.6 ± 21.52

Metabolism	Normal ranges	2 weeks				4 weeks			
		Vehicle	GSK	VEN	GSK+VEN	Vehicle	GSK	VEN	GSK+VEN
Glucose (mg/dL)	106.74 - 353.74	206.75 ± 54.36	218.75 ± 64.1	228.75 ± 15.41	235 ± 31.95	204 ± 21.3	169.8 ± 94.09	224.6 ± 11.84	256.2 ± 31.28
Total Protein (g/dL)	4.48 - 6.32	6.05 ± 0.37	6.25 ± 0.58	5.75 ± 0.13	5.98 ± 0.36	5.66 ± 0.09	5.74 ± 0.21	5.68 ± 0.13	5.84 ± 0.24
Cholesterol (mg/dL)	52.62 - 167.47	135 ± 19.37	143.5 ± 26.49	106.75 ± 15.59	130.25 ± 12.69	1.58 ± 0.04	1.46 ± 0.15	1.52 ± 0.08	1.58 ± 0.08
Albumin (g/dL)	3.60 1.92 - 4.11	3.53 ± 0.17	3.73 ± 0.28	3.55 ± 0.06	3.73 ± 0.19	3.46 ± 0.05	3.4 ± 0.16	3.44 ± 0.11	3.6 ± 0.14
Globulin (g/dL)	0.92 - 3.64	2.53 ± 0.25	2.53 ± 0.31	2.2 ± 0.12	2.25 ± 0.21	2.2 ± 0.07	2.34 ± 0.17	2.24 ± 0.05	2.26 ± 0.13
Alb/Glob Ratio		1.4 ± 0.14	1.45 ± 0.1	1.63 ± 0.1	1.68 ± 0.15	117.8 ± 21.16	118 ± 8.72	128.4 ± 10.83	118 ± 14.09

All values are presented as mean ± SD (n=4-5) with corresponding normal reference ranges for mice.

Supplementary Table 3: Complete blood count and leukocyte differentials in mice treated with vehicle, GSK, VEN, or the GSK+VEN combination for 2 or 4 weeks.

CBC with differentials	Normal ranges	2 weeks				4 weeks			
		Vehicle	GSK	VEN	GSK+VEN	Vehicle	GSK	VEN	GSK+VEN
RBC (M/ μ L)	6.36-9.42	9.47 $\pm$ 0.11	9.62 $\pm$ 0.47	9.6 $\pm$ 0.34	8.9 $\pm$ 1.8	8.86 $\pm$ 0.52	8.52 $\pm$ 0.58	8.73 $\pm$ 0.51	8.05 $\pm$ 1.63
Hb (g/dL)	11-15.1	13.2 $\pm$ 1.23	13.68 $\pm$ 1.64	13.38 $\pm$ 0.54	12.6 $\pm$ 2.38	11.48 $\pm$ 0.61	11.42 $\pm$ 1.15	11.28 $\pm$ 0.54	10.36 $\pm$ 2.4
HCT (%)	35.1-45.4	49.48 $\pm$ 1.85	51.08 $\pm$ 3.8	47.53 $\pm$ 2.04	44.98 $\pm$ 7.94	46.04 $\pm$ 2.21	44.88 $\pm$ 3.28	44.7 $\pm$ 1.7	41.16 $\pm$ 8.08
MCV (fL)	45.4-60.3	52.25 $\pm$ 1.73	53.13 $\pm$ 2.12	49.5 $\pm$ 0.98	50.7 $\pm$ 1.5	52.04 $\pm$ 2.46	52.66 $\pm$ 1.08	51.3 $\pm$ 1.28	51.26 $\pm$ 2.71
MCH (pg)	14.1-19.3	13.93 $\pm$ 1.4	14.18 $\pm$ 0.99	13.95 $\pm$ 0.26	14.18 $\pm$ 0.26	12.96 $\pm$ 0.71	13.34 $\pm$ 0.52	12.94 $\pm$ 0.55	12.8 $\pm$ 0.98
MCHC (g/dL)	30.2-34.2	26.68 $\pm$ 2.37	26.73 $\pm$ 2.05	28.15 $\pm$ 0.76	27.98 $\pm$ 0.43	24.94 $\pm$ 0.48	25.42 $\pm$ 1.24	25.24 $\pm$ 0.82	24.98 $\pm$ 1.38
RDW (%)	12.4-27	17.05 $\pm$ 0.13	18.83 $\pm$ 1.13	17.25 $\pm$ 0.26	18.4 $\pm$ 1.29	17 $\pm$ 0.33	18.26 $\pm$ 0.67	19.56 $\pm$ 1.58	21.3 $\pm$ 1.79
MPV (fL)	5.0-20.2	5.18 $\pm$ 0.24	5.33 $\pm$ 0.22	5.28 $\pm$ 0.17	5.58 $\pm$ 0.21	5.06 $\pm$ 0.09	5.32 $\pm$ 0.19	5.32 $\pm$ 0.18	5.38 $\pm$ 0.11
PLT (K/ μ L)	592-2972	766.25 $\pm$ 126.74	789.75 $\pm$ 143.19	1070 $\pm$ 93.82	1020 $\pm$ 140.09	611.6 $\pm$ 196.51	536.2 $\pm$ 304.42	762.2 $\pm$ 327.44	502.2 $\pm$ 343.7
WBC (K/ μ L)	1.8-10.7	9.39 $\pm$ 1.87	6.36 $\pm$ 2.58	3.74 $\pm$ 0.61	2.94 $\pm$ 0.46	9.49 $\pm$ 2.03	12.12 $\pm$ 3.97	8.99 $\pm$ 3.91	2.94 $\pm$ 0.46
NE (K/ μ L)	0.1-2.4	2.05 $\pm$ 0.89	1.1 $\pm$ 0.64	1.02 $\pm$ 0.25	0.8 $\pm$ 0.08	1.75 $\pm$ 0.73	2.14 $\pm$ 0.94	2.02 $\pm$ 1.02	0.8 $\pm$ 0.08
LY (K/ μ L)	0.9-9.3	6.3 $\pm$ 1.23	4.54 $\pm$ 1.81	2.39 $\pm$ 0.33	1.66 $\pm$ 0.65	6.41 $\pm$ 1.25	8.29 $\pm$ 2.47	5.58 $\pm$ 2.35	1.66 $\pm$ 0.65
MO (K/ μ L)	0-0.4	0.67 $\pm$ 0.24	0.52 $\pm$ 0.16	0.34 $\pm$ 0.05	0.25 $\pm$ 0.05	0.67 $\pm$ 0.15	0.91 $\pm$ 0.37	0.7 $\pm$ 0.32	0.25 $\pm$ 0.05
EO (K/ μ L)	0-0.2	0.29 $\pm$ 0.22	0.18 $\pm$ 0.09	0.11 $\pm$ 0.05	0.19 $\pm$ 0.29	0.54 $\pm$ 0.22	0.62 $\pm$ 0.28	0.52 $\pm$ 0.29	0.19 $\pm$ 0.29
BA (K/ μ L)	0-0.2	0.08 $\pm$ 0.05	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.05 $\pm$ 0.06	0.11 $\pm$ 0.07	0.15 $\pm$ 0.1	0.16 $\pm$ 0.09	0.05 $\pm$ 0.06

All values are presented as mean $\pm$ SD (n=4-5) with corresponding normal reference ranges for mice.

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