

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

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Abstract

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy requiring concomitant targeting of critical cellular survival pathways due to resistance and frequent relapse with monotherapies. Venetoclax (VEN), a BCL-2 inhibitor, is one such promising clinical agent best utilized in combination therapies due to transient responses and acquired resistance. Given the involvement of the Rho/ROCK pathway in VEN activity, we combined Rho-associated coiled-coil-containing protein kinase inhibitors (ROCKi) with VEN to achieve superior antileukemic activity. The ROCKi (Fasudil, DJ4, GSK269962A) synergized with VEN to enhance cytotoxicity in both VEN-sensitive and VEN-resistant cell lines *in vitro*. Among the three ROCKi, GSK269962A (GSK) was best-tolerated in combination with VEN and effectively inhibited leukemia growth across multiple AML cell line-derived xenograft models *in vivo*. The GSK+VEN combination exhibited additive to synergistic cytotoxicity in primary AML patient cells *ex vivo* and enhanced antileukemic activity in a patient-derived xenograft model. Additionally, the GSK+VEN combination significantly decreased the clonogenicity of primary AML cells, relatively sparing normal cells. Functional assays demonstrated enhanced apoptosis (Annexin V, caspase-3/7), elevated reactive oxygen species, and mitochondrial depolarization in both VEN-sensitive and VEN-resistant AML cells following combination treatment. Mechanistically, GSK augmented venetoclax responses by down-regulating anti-apoptotic proteins (BCL2, MCL1) and inducing pro-apoptotic mediators (NOXA, MCL1 short isoforms), including in VEN-resistant AML cells. Together, these findings across multiple preclinical AML models demonstrate synergistic antileukemic activity and support combining VEN with ROCKi as a promising therapeutic strategy for AML.

Introduction

Acute myeloid leukemia (AML) remains among the most lethal leukemias, with a 5-year overall survival rate of only

32.9%.¹ Despite significant research efforts, improvements in AML survival outcomes over the past two decades have been limited. Only a small proportion of patients can tolerate intensive chemotherapy or allogeneic stem cell

transplantation.²⁻⁴ Though there have been significant advances in lower-intensity therapy options in recent years,^{3,4} further efforts are needed to achieve improved remission and survival rates among older adults and patients who are unfit for intensive chemotherapy.

The approval of the anti-apoptotic B-cell lymphoma 2 (BCL-2) inhibitor venetoclax (VEN; also known as ABT-199) in combination with hypomethylating agents or low-dose cytarabine (LDAC) was a significant milestone for AML patients ineligible for intensive chemotherapy, both in the frontline and relapsed or refractory (R/R) settings. While VEN-based regimens achieve response rates of approximately 54-74% in newly diagnosed AML, these rates decline to 20-40% in patients with R/R AML.^{3,5-8} VEN combination regimens are not curative, and nearly all patients develop resistance to VEN and are refractory or progress within two years.⁷ Therefore, there is a critical unmet need for effective, well-tolerated treatment strategies that can enhance response rates to VEN in AML patients.

To better understand VEN resistance, Chen et al.⁹ performed a genome-wide CRISPR/Cas9 loss-of-function screen with VEN in MOLM-13 AML cells and identified several “sensitizer” genes (i.e., associated with improved VEN response), including the Rho/ROCK (Rho-associated protein kinase) pathway. Similarly, ROCK1 was elevated in the secretome of AML patients with high anti-apoptotic index.¹⁰ More recent work has demonstrated that rho-kinases ROCK1/2 are up-regulated in AML cells that are resistant to VEN and doxorubicin.^{11,12} These findings suggest that rho-kinases play an antiapoptotic role, and inhibition of ROCK activity may synergize with VEN and overcome VEN resistance to effectively induce apoptotic cell death in AML.

The Rho/ROCK signaling pathway regulates actin cytoskeletal dynamics, cell migration, and various cellular processes including cell proliferation, differentiation, survival, and apoptosis.^{13,14} ROCK1/2, Ser/Thr protein kinases, have aided in the development and progression of various cancers, including AML, and drug resistance.^{11-13,15,16} Over the last decade, ROCK inhibitors (ROCKi) such as fasudil, DJ4, and GSK269962A (GSK) have been explored as potential therapeutic agents for AML.¹⁷⁻¹⁹ However, the use of ROCKi in combination therapy for AML remains largely underexplored. Here, we evaluated the activity of ROCKi in combination with VEN for the effective treatment of AML and to circumvent VEN-resistance. The combination synergistically induced cytotoxicity in AML cell lines *in vitro* and primary AML patient samples *ex vivo*. The addition of GSK enhanced VEN activity by down-regulating survival proteins and promoting the expression of proapoptotic proteins in both naïve and VEN-resistant AML cells. *In vivo* results show that GSK potentiated the efficacy of VEN in multiple cell line-derived xenografts (CDX) and patient-derived xenograft (PDX) model. These findings provide strong preclinical evidence to support the therapeutic potential of VEN and ROCKi combination in AML.

Methods

Cell culture

Acute myeloid leukemia cell lines and primary AML patient cells were cultured as previously described.²⁰ Cell lines were validated by short tandem repeat (STR) profiling (Genetica). See the *Online Supplementary Appendix* for details of reagents.

Clinical samples

Bone marrow aspirates or peripheral blood samples were obtained from AML patients after informed consent using protocols approved by the Institutional Review Board (IRB# 2000-186) of Penn State College of Medicine. Mutational profiling was performed as described previously.²¹ See the *Online Supplementary Appendix* for more details.

Cell viability assays and synergy analysis

The cell viability of AML cells after combinatorial treatment was assessed by MTS assay.¹⁸ The viability of primary AML patient cells was measured using CellTiter-Glo luminescence assay (Promega). The data were represented as % viability relative to vehicle control. The synergy between two drugs was analyzed using the SynergyFinder 2.0 web-application tool (<https://synergyfinder.fimm.fi/>).²² See the *Online Supplementary Appendix* for more details.

Assays and other methods

The details of colony-forming assay and other functional assays, including Annexin V staining, caspase-3/7 activity, reactive oxygen species (ROS) measurements, and mitochondrial membrane potential ($\Delta\Psi_m$) are provided in the *Online Supplementary Appendix*. Methods for assessing cellular respiration and $\Delta\Psi_m$ in intact cells are also described in the *Online Supplementary Appendix*.

Immunoblotting analysis

The AML cell lines were left untreated or treated with DMSO (vehicle), GSK, VEN, and GSK+VEN combination at the indicated doses and time. Whole cell lysates were subjected to immunoblotting analysis with respective primary antibodies as described in the *Online Supplementary Appendix*.

In vivo mice efficacy and safety studies

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC). The efficacy of GSK and VEN, alone and in combination, was evaluated in CDX and PDX models as described previously.^{18,20} Short- and long-term safety studies were conducted, full experimental details of all *in vivo* studies are provided in the *Online Supplementary Appendix*.

Statistical analysis

Statistical tests were performed using GraphPad Prism 10 software. Results are presented as the mean $\pm$ standard

error of the mean (SEM) or standard deviation (SD) from 3 independent experiments. $P<0.05$ (95% CI) is considered statistically significant. Comparisons between two groups were performed using a t test, and comparisons among three or more groups were made by analysis of variance (ANOVA) with appropriate post hoc tests. The *Online Supplementary Appendix* contains more detailed information on the experimental methods and materials.

Results

ROCK inhibitors synergize with venetoclax to induce potent anti-leukemic effects in acute myeloid leukemia cell lines *in vitro*

Since Rho/ROCK pathway genes were negatively enriched in the CRISPR loss-of-function screen with VEN (*Online Supplementary Figure S1A, B*),⁹ we performed correlation analysis to establish the relationship between VEN sensitivity (AUC-area under the curve) and normalized expression levels of Rho/ROCK pathway genes using the Beat AML dataset (<http://vizome.org/aml2/inhibitor/>).²³ The expression of Rho/ROCK pathway genes except *ROCK2* showed a positive correlation (higher expression linked to resistance) with VEN activity (*Online Supplementary Figure S1C*). The positive correlation of *ROCK1* levels was comparable to that of *MCL1*, a well-known anti-apoptotic protein associated with VEN resistance (*Online Supplementary Figure S1D*). These findings support our hypothesis that suppressing ROCK activity can potentiate VEN-induced cytotoxicity in AML cells. To test the potential utility of ROCKi in combination with VEN, a cytotoxicity assay was performed in VEN-sensitive and VEN-resistant

AML cells, followed by Bliss analysis to assess the nature of the interaction. All the tested ROCKi (Fasudil, DJ4, and GSK269962A) exhibited strong synergistic cytotoxicity in combination with VEN in the VEN-sensitive AML cell lines (MV4-11, MOLM-13, HL-60, and OCI-AML2) tested (Table 1, Figure 1A). Inherently VEN-resistant (OCI-AML3, KG-1, and U937) or acquired VEN-resistant (MV4-11/VENR) AML cells exhibited at least additive (often synergistic) growth inhibition with the ROCKi and VEN combination (Table 1, Figure 1B). Representative dose-response matrices and corresponding Bliss synergy analysis for the ROCKi+VEN combination *versus* MV4-11 cells (naïve and VEN-resistant) are shown in Figure 1 and *Online Supplementary Figure S2*. These findings show that inhibition of ROCK activity significantly enhances VEN-induced cytotoxicity in both VEN-sensitive and VEN-resistant AML cells *in vitro*.

GSK269962A synergizes with venetoclax to reduce leukemia burden in mice

We next assessed the *in vivo* efficacy of different ROCKi (Fasudil, DJ4, and GSK) in combination with VEN using a luciferase-labeled MV4-11 (sensitive to GSK and VEN) CDX model (Figure 2A, *Online Supplementary Figure S3A*). Of the three ROCKi tested, GSK exhibited strong synergistic antileukemic activity in combination with VEN *in vivo*. Mice treated with both GSK (10 mg/kg) and VEN (100 mg/kg) for three weeks demonstrated a significantly lower leukemia burden compared to vehicle control (Figure 2B, C). Flow cytometric analysis of peripheral blood also revealed that mice treated with GSK+VEN combination had significantly lower burdens of circulating leukemia cells (hCD45+Ve) compared to vehicle control (Figure 2D). Moreover, mice

Table 1. Bliss synergy scores for the ROCK inhibitors (DJ4, Fasudil, GSK269962A) and venetoclax combination in acute myeloid leukemia cell lines.

AML cell line	VEN activity	Bliss synergy score			Mutated genes	TP53 status
		DJ4 + VEN	Fasudil + VEN	GSK + VEN		
MV4-11	Sensitive	15.89 [§]	12.24 [§]	13.49 [§]	<i>FLT3</i> -ITD, <i>KMT2A</i> r, <i>MLL</i> -AF4	WT
MV4-11/VENR	Resistant	29.72 [§]	13.97 [§]	10.44 [§]	-	-
MOLM-13	Sensitive	10.91 [§]	12.62 [§]	10.52 [§]	<i>FLT3</i> -ITD, <i>KMT2A</i> r	WT
HL-60	Sensitive	19.24 [§]	11.57 [§]	25.59 [§]	<i>CDKN2A</i> , <i>NRAS</i>	del
OCI-AML2	Sensitive	18.76 [§]	12.38 [§]	18.21 [§]	<i>DNMT3A</i>	WT
OCI-AML3	Resistant	18.69 [§]	12.81 [§]	-3.07 [#]	<i>DNMT3A</i> , <i>NPM1</i> , <i>NRAS</i>	WT
U937	Resistant	10.94 [§]	3.43 [#]	9.4 [#]	<i>PTEN</i> , <i>PTPN11</i> , <i>WT1</i>	Mut
KG-1	Resistant	22.32 [§]	14.5 [§]	7.81 [#]	<i>NRAS</i>	Mut

*< -10: antagonistic; # -10 to 10: additive; [§]>10: synergistic. AML: acute myeloid leukemia; GSK: GSK269962A; Mut: mutated; VEN: venetoclax; VENR: VEN resistant; WT: wild-type.

treated with GSK+VEN combination showed significantly higher overall median survival of 56 days ($P<0.01$), relative to the single agent (approx. 45 days) and vehicle (38 days) groups (Figure 2E). Further testing of VEN (100 mg/kg) ef-

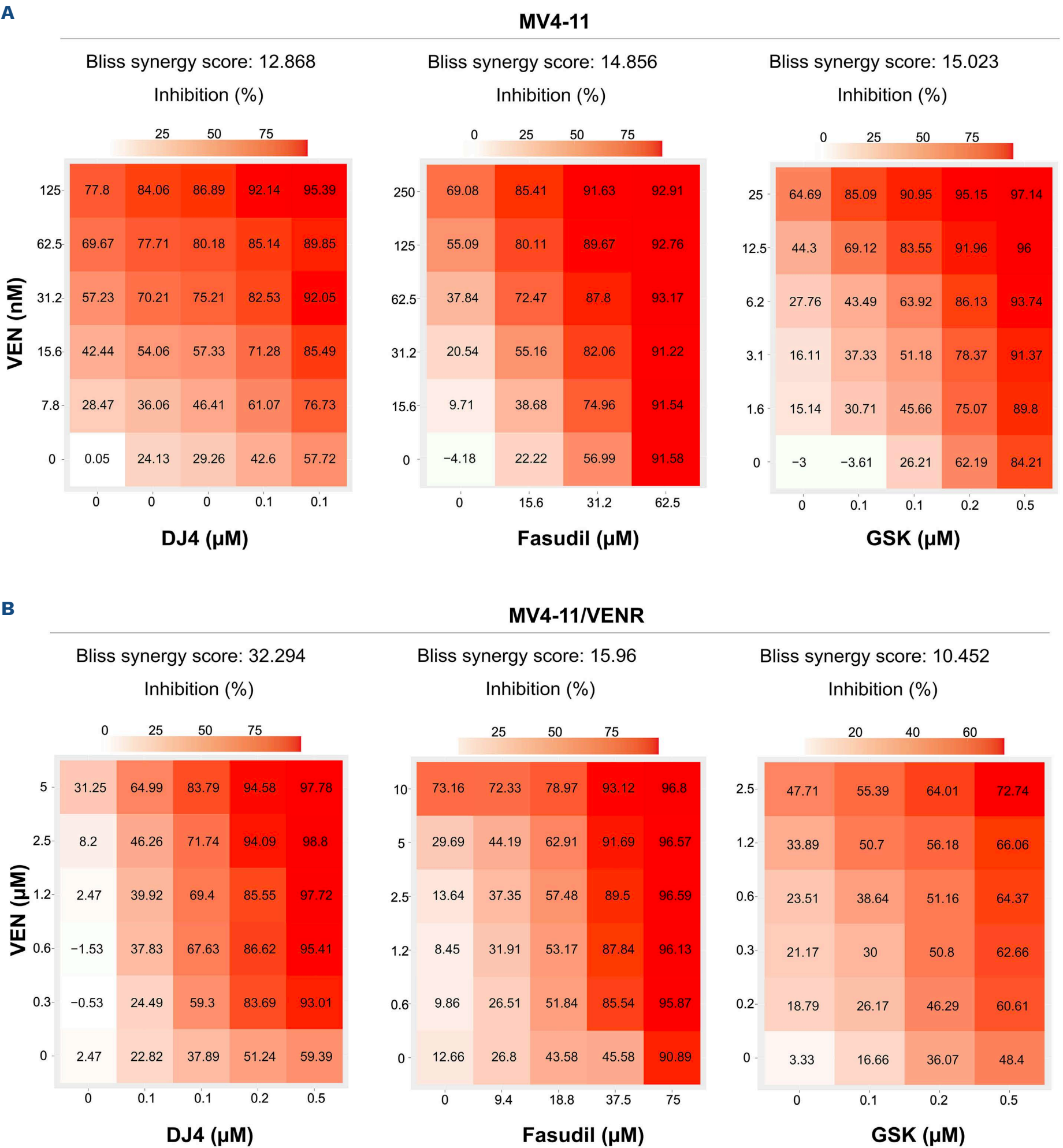
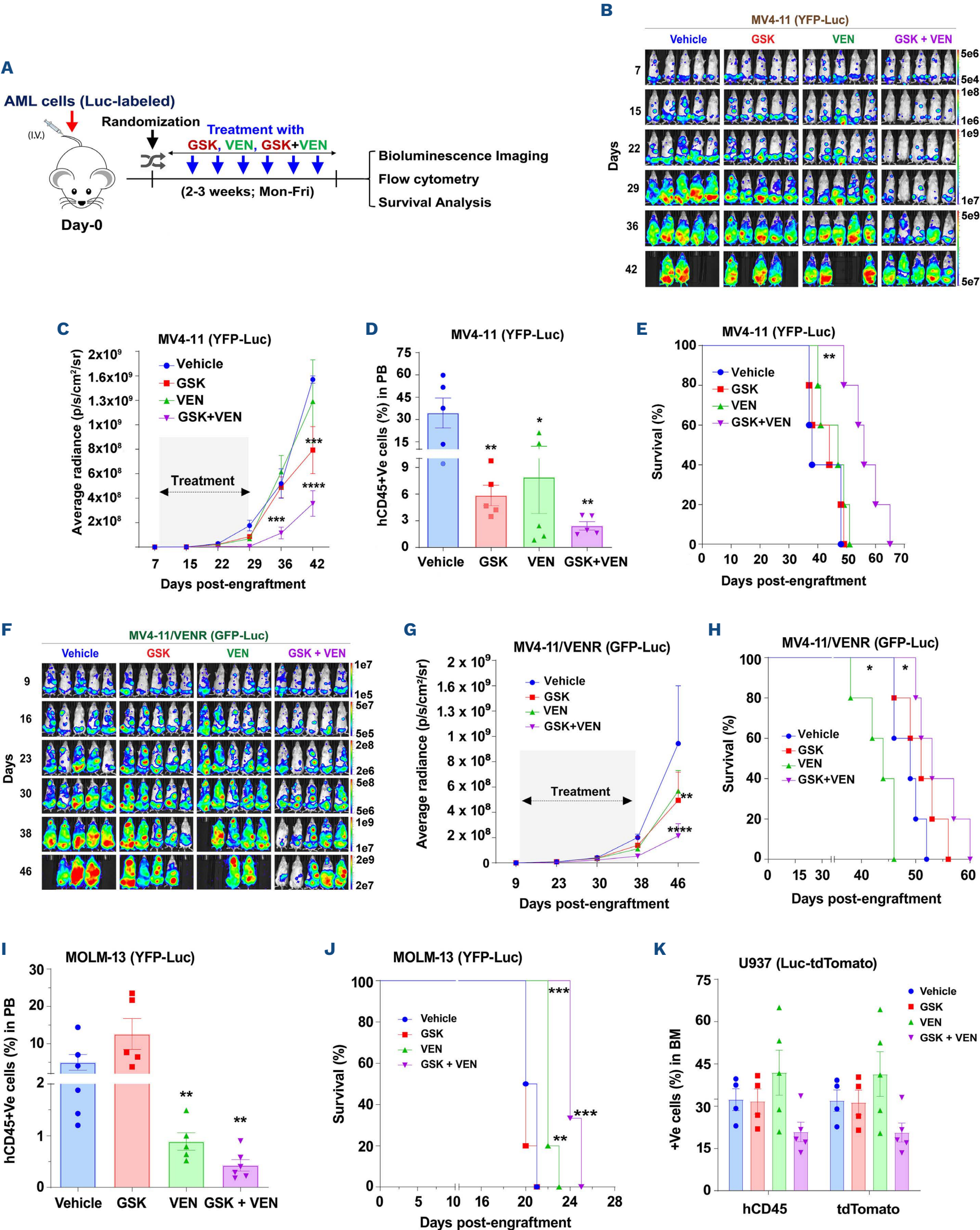


Figure 1. ROCK inhibitors exhibit synergistic cytotoxicity with venetoclax combination in acute myeloid leukemia cell lines *in vitro*. (A and B) Dose-response matrix showing the percent growth inhibition in (A) MV4-11 and (B) MV4-11/venetoclax (VEN) resistant (VENR) cells along with Bliss synergy score after ROCKi and VEN combination treatment for 24 hours (DJ4, Fasudil) and 48 hours (GSK269962A-GSK). Bliss scores greater than 10 were considered synergistic. Representative data from at least 3 independent experiments are presented.



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Figure 2. GSK269962A and venetoclax combination synergistically decreases leukemia burden in acute myeloid leukemia cell line xenograft models. (A) Schematic depicting the treatment plan in cell line-derived xenograft (CDX) mouse models. The NRG-S mice were intravenously injected with acute myeloid leukemia (AML) cells (MV4-11-YFP-Luc, MV4-11/VENR-GFP-Luc, MOLM13-YFP-Luc, and U937-Luc-tdTomato) and randomized into experimental groups (Vehicle, GSK269962A [GSK], venetoclax [VEN], and GSK+VEN) based on bioluminescence imaging (BLI) signals before the initiation of treatment. The leukemia progression was monitored through BLI imaging, flow cytometry, and survival analysis. (B) BLI of MV4-11-YFP-Luc CDX mice (N=5) at indicated days. (C) The leukemia burden in the MV4-11-YFP-Luc mice was quantified in the terms of BLI signals and presented as average radiance (p/s/cm²/sr). *** $P < 0.001$, **** $P < 0.0001$ by 2-way ANOVA (Tukey's multiple comparisons test). (D) Flow cytometric analysis of peripheral blood (PB) from MV4-11-YFP-Luc CDX mice at 36 days post engraftment to assess circulating human leukemic cells (% hCD45 positive cells). Data are presented as mean $\pm$ standard error of mean (SEM) (N=5). ** $P < 0.01$ by one-way ANOVA (Tukey's multiple comparisons test) compared to vehicle control. (E) Kaplan-Meier survival analysis of MV4-11-YFP-Luc CDX mice after treatment with GSK, VEN and GSK+VEN combination. ** $P < 0.01$ by Gehan-Breslow-Wilcoxon test (N=5). (F) The MV4-11/VENR-GFP-Luc CDX mice (N=5) were monitored regularly by an *in vivo* imaging system at indicated days. (G) The leukemia burden in the MV4-11/VENR-GFP-Luc CDX mice after treatment with GSK, VEN and GSK+VEN combination 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). (H) Kaplan-Meier survival analysis of MV4-11/VENR CDX mice after treatment with GSK, VEN and GSK+VEN combination. * $P < 0.05$ by Gehan-Breslow-Wilcoxon test indicates statistical significance compared to vehicle control. (I) Flow cytometric analysis of PB collected from MOLM-13-YFP-Luc CDX mice after 20 days of post engraftment for the detection of circulatory human leukemic cells (% hCD45 positive cells). Data are presented as mean $\pm$ SEM (N=5-6). ** $P < 0.01$ by 1-way ANOVA (Tukey's multiple comparisons test) compared to GSK. (J) Kaplan-Meier survival analysis of MOLM-13-YFP-Luc CDX mice after treatment with GSK, VEN and GSK+VEN combination. ** $P < 0.01$, *** $P < 0.001$ by Gehan-Breslow-Wilcoxon test (N=5-6). (K) Flow cytometric analysis of BM cells collected from U937-Luc-tdTomato engrafted CDX mice after 21 days post transplant for the detection of human leukemic cells (% tdTomato and hCD45 positive cells). Data are presented as mean $\pm$ SEM (N=4-5).

ficacy in combination with fasudil (25 mg/kg) or DJ4 (10 mg/kg) showed minimal or no advantage over single agents alone due to poor pharmacokinetics and resulted in early deaths because of off-target toxicity of fasudil and DJ4 (*Online Supplementary Figure S3*). Given its ROCK selectivity, oral bioavailability, and apparent tolerability, most subsequent efforts were focused on the combination of GSK with VEN.

Next, the combination of GSK with VEN was tested in other CDX mouse models: MV4-11/VENR (acquired VEN-resistance), MOLM-13 (inherently GSK-resistant), and U937 (inherently resistant to both GSK and VEN) (Figure 2, *Online Supplementary Table S1*). The MV4-11/VENR CDX mice treated with GSK+VEN combination showed significantly lower leukemia burden (Figure 2F, G) and prolonged overall median survival to 53 days from 49 days for vehicle and 44 days for VEN subjects (Figure 2H). Similarly, MOLM-13 CDX mice treated with the GSK (10 mg/kg) and VEN (50 mg/kg) combination showed a significantly lower leukemia burden (Figure 2 I, *Online Supplementary Figure S4A, B*) and increased overall survival to 24 days from 20 days for vehicle or GSK and 22 days for VEN subjects (Figure 2J). Flow cytometric analysis showed that GSK treatment alone is ineffective but can significantly lower the percentage of circulating leukemia cells in combination with VEN (Figure 2 I). Lastly, we tested the effectiveness of GSK+VEN combination in the U937 CDX model. Similar to MOLM-13, U937 CDX is an aggressive leukemia model and showed a significantly lower leukemia burden after two weeks of treatment with the GSK (10 mg/kg) and VEN (100 mg/kg) combination (*Online Supplementary Figure S4C, D*). Flow cytometric analysis of bone marrows revealed that GSK+VEN group exhibited lower engraftment levels compared to vehicle and single agent groups (Figure 2K). Altogether, our

in vivo efficacy studies indicate that the selective ROCK1 GSK potentiates VEN-mediated antileukemic activity in naïve and VEN-resistant (inherent or acquired) preclinical AML CDX mouse models.

Venetoclax synergizes with GSK269962A against primary acute myeloid leukemia patient cells and cooperatively decreases leukemic burden in a patient-derived xenograft mouse model

To validate cell line findings, we analyzed multiple primary AML patient samples with diverse genetic alterations. Bliss analysis revealed that more than 60% of the tested AML patient cells showed synergy with the GSK+VEN combination, whereas the remaining cases exhibited additive responses (Table 2, Figure 3A, B, *Online Supplementary Figure S5*). This effect shows no clear correlation with major AML subgroups (*Online Supplementary Figure S6*). The addition of GSK to VEN significantly reduced clonogenicity of leukemic progenitor cells compared to single-agent treatments (Figure 3C). Assessment of GSK+VEN combination on normal cord-blood mononuclear cells (CB-MNC) showed less inhibition of colony formation relative to AML patient cells (Figure 3C). These findings indicate that the GSK+VEN combination effectively exerts cytotoxicity in clinically relevant primary AML cells, inhibiting their clonogenicity while relatively sparing normal hematopoietic progenitors.

We next assessed the *in vivo* efficacy of GSK+VEN combination in a clinically relevant AML bioluminescent PDX mouse model (Figure 4A). A luciferase-labeled PDX model was generated to evaluate the combination therapy and monitor leukemia progression. Notably, the luciferase-labeled 1265 cells used for PDX injection (passage 2) exhibited mutation allele frequencies comparable to those of the primary cells (passage 0).²⁴ The treatment of 1265-Luc PDX mice with GSK

Table 2. Characteristics of primary acute myeloid leukemia patient cells are listed along with the corresponding Bliss synergy scores for GSK269962A and venetoclax combination.

Identifier	Disease stage	Age (yr)/ Gender	WBC x10 ⁹ /L	Blast %	Cytogenetics	Molecular data	Bliss synergy score
1259	Refractory	79/F	81.30	90.5	43,XX,add(2)(q31),del(2)(q11.2),3,del(5)(q13q33),add(6)(p23),add(6)(q21),add(7)(q11.2),add(14)(p11.2) add(14)(p11.2),del(17)(p11.2),-20,-21[11]	TP53	28.23
215	Newly diagnosed	74/M	348.00	94.0	44,XY,add(9)(p13), -16,-17,+mar[cp3]	DNMT3A	19.15
1364	Newly diagnosed	76/M	168.00	85.0	46,XY,t(1;4)(q25;p16)?c[20]	NPM1, FLT3-ITD, DNMT3A	17.44
1244	Newly diagnosed	51/M	138.90	63.5	46,XY,t(3;5)(q25;q35)[20]	FLT3-ITD	17.24
1258	Refractory	59/F	18.24	11.0	43-44,X,der(X)t(X;9)(q22;q22),add(2)(q31),add(3)(p21),del(3)(q11.1),add(4)(p14),add(5)(q11.2),-7,+8,-10,11,add(12)(p11.2),add(14)(q22),-17,-17,der(18)t(17;18)(q11.2;q23),add(19)(q13.3),add(20)(q11.2),+1-3mar[cp17]/84-89,idemx2[cp3]	TP53	14.94
1256	Newly diagnosed	50/F	61.38	95.7	46,XX[20]	NPM1, FLT3-ITD, DNMT3A, IDH2	13.33
1284	Relapsed	68/F	55.00	93.0	47,XX,+13[18]/46,XX,t(7;15)(q34;q11.2),del(13)(q12q14)	DNMT3A, IDH2, NRAS, SRSF2, RUNX1, STAG2	13.14
1265*	Newly diagnosed	74/M	217.36	96.0	46,XY,t(7;11)(p15;p15)[20]	HOXA9/ NUP98 fusion, TP53, TET2, BLM	12.92
1279	Relapsed	57/M	80.40	88.0	46,XY[20]	NPM1, DNMT3A, IDH2, PTPN11	12.49
1006*	Newly diagnosed	48/M	22.04	73.8	46,XY,add(5)(q31)[11]/46,XY[9]	TET2, CEBPA, IDH1, GATA2, NOTCH1	12.3
329	Newly diagnosed	72/F	41.90	75.0	45,XX,inv(3)(q21q26.2),-7 [20]	KRAS, CRLF2	11.75
1032	Relapsed	48/F	15.85	74.5	46,XX[20]	NPM1, FLT3-TKD, IDH1, DNMT3A	11.52
1361	Newly diagnosed	59/F	114.00	90.0	46,XX[20]	ASXL1, FLT3-ITD, WT1	9.03
1232	Relapsed	46/F	46.40	52.8	46,XX[20]	FLT3-ITD, TET2, CEBPA	8.79
1342	Refractory	27/M	139.70	86.0	45,X,-Y[20]	NPM1, TET2	8.66

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Identifier	Disease stage	Age (yr)/ Gender	WBC x10 ⁹ /L	Blast %	Cytogenetics	Molecular data	Bliss synergy score
1229	Relapsed	57/F	21.36	0.92	46,XX[20]	NRAS, KRAS, IDH1, DNMT3A, ASXL1, STAG2	7.63
1341	Newly diagnosed	83/M	201.00	89.0	46,XY,t(1;3)(p34.1;q27),t(2;18)(q31;q11.2),del(11)(p11.2p15)[20]	CBL, KIT	7.51
939*	Newly diagnosed	45/F	364.58	93.8	46,XX[20]	FLT3-ITD, BCORL1, SMG1, WT1	5.33
1290	Newly diagnosed	74/M	99.07	61.0	46,XY[20]	ASXL1, DNMT3A, IDH1, KRAS, NRAS, RUNX1	4.66
1044*	Newly diagnosed	70/M	36.40	63.8	46,XY,i(17)(q10)[12]/47,idem,+13[2]/47,XY,+mar[6]	ASXL1, SRSF2, CBL, APC, SETBP1	4.64
1240*	Newly diagnosed	64/F	116.50	70.0	41,XX,-5,add(6)(q13),der(7;16)(p10;q10),-11,-16,-17,add(17)(p11.2),-18,add(21)(p11.2),add(22)(p11.2)[18]/46,XX[2]	NPM1 neg, FLT3 neg, and CEBPA neg. Others not tested	Used only in CFU assay
1103*	Newly diagnosed	41/F	247.25	86.0	46,XX[20]	PTPN11, FLT3-ITD, NOTCH1	

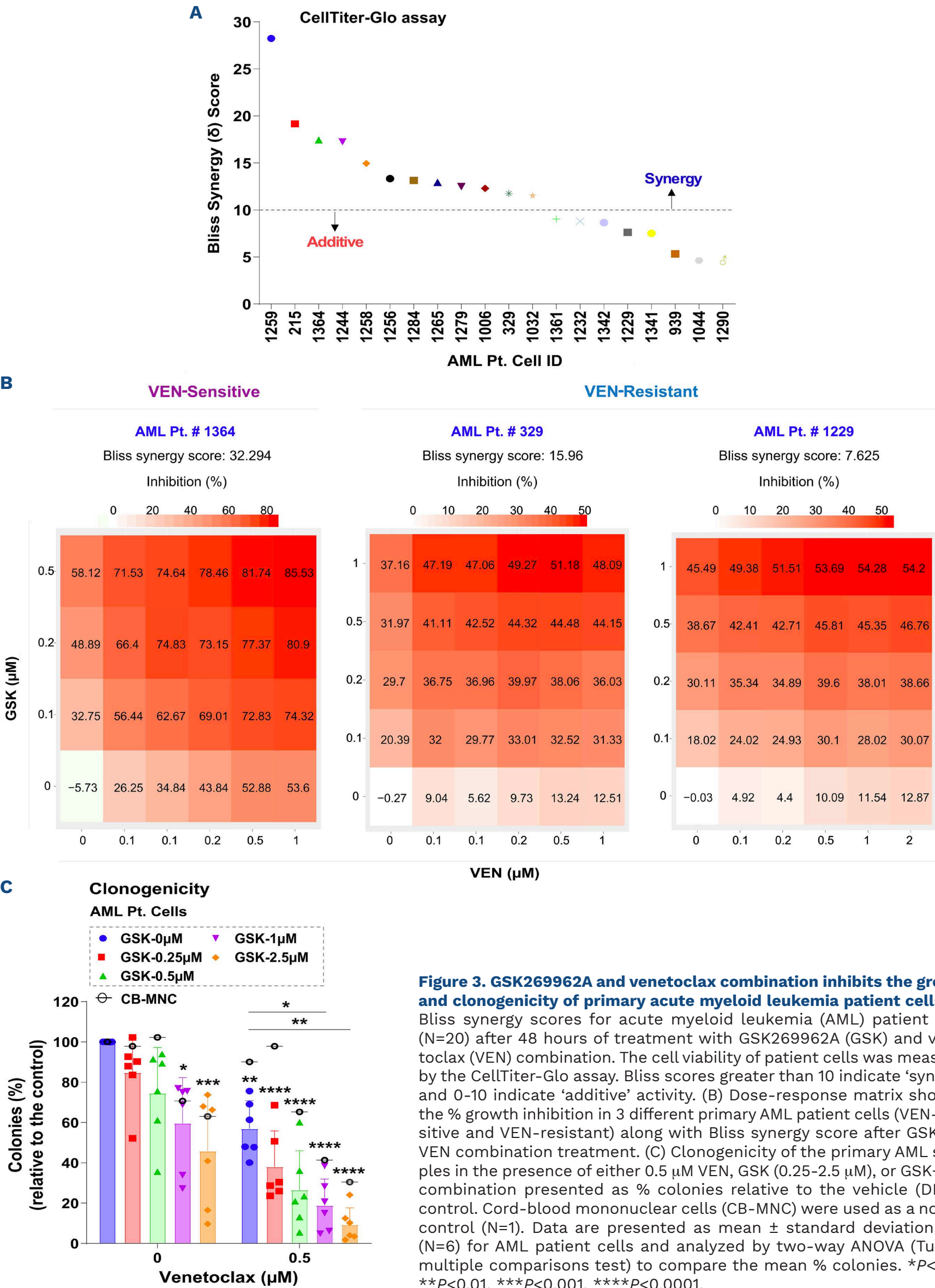
*Identifier indicates the cells used for colony-forming unit (CFU) assay. F: female, M: male; neg.: negative; WBC: white blood cells; yr: years.

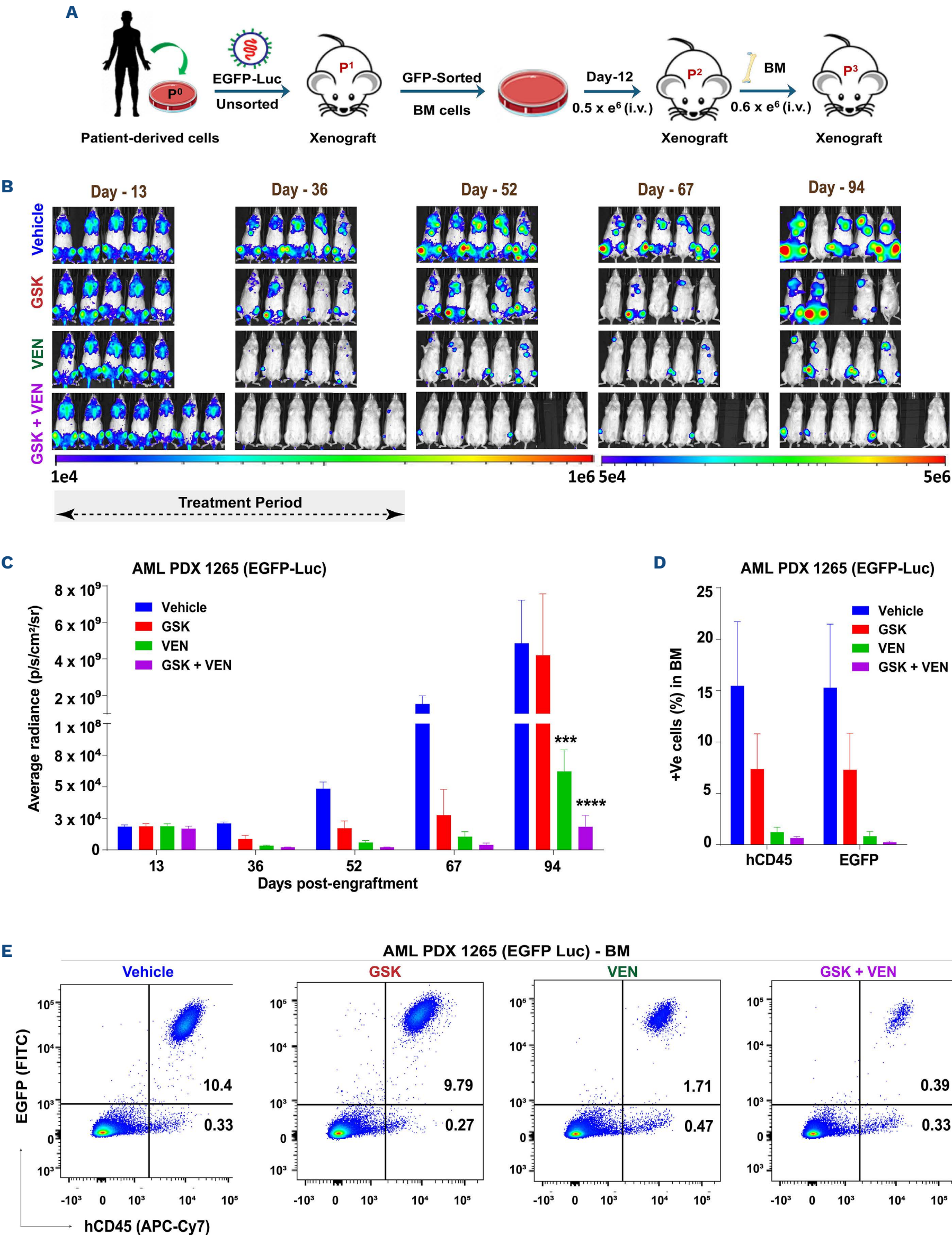
(30 mg/kg) and VEN (50 mg/kg) combination resulted in a decreased leukemic burden compared to vehicle control (Figure 4B, C). Engraftment analysis by flow cytometry revealed that PDX mice treated with GSK+VEN combination exhibited a lower leukemia burden compared to vehicle group (Figure 4D, E). Notably, in the PDX study, 2 deaths were observed, one in the GSK group and one in the GSK+VEN group for unknown reasons. No deaths or treatment-related toxicity were observed across four CDX models treated for two to nearly four weeks, nor in the 2- and 4-week safety studies. The affected NRG-S mice appeared clinically normal, with stable bioluminescent signals and no gross abnormalities at necropsy. These results suggest that the GSK+VEN combination provides antileukemic activity that is superior to that of VEN for the treatment of AML.

GSK269962A, venetoclax, and their combination were well tolerated following short- and long-term exposure, with no observable signs of treatment-related toxicity

Although the GSK+VEN combination was well-tolerated across leukemia-bearing animal models, 2 deaths in the PDX study prompted dedicated short- and long-term safety evaluations. Normal Swiss Webster mice were treated for

two weeks (short-term) or four weeks (long-term) with GSK (30 mg/kg) and VEN (50 or 100 mg/kg), alone or in combination (*Online Supplementary Figures S7A, 8A*). Body weight increased similarly across all groups, with no indication of treatment-related weight loss or overt clinical toxicity (*Online Supplementary Figures S7B, 8B*). Mild reductions in total white blood cell counts were noted but remained within normal limits; lymphopenia was restricted to the GSK+VEN group, whereas neutrophils, monocytes, erythrocyte indices, hemoglobin, and platelets were unchanged apart from the expected VEN-associated platelet increase (*Online Supplementary Table S2, Online Supplementary Figures S7C, 8C*). Serum biochemical markers of liver and kidney function, electrolytes, metabolic parameters, and calcium also remained within the normal range with slight increases in blood glucose that remained within the normal range and mild elevations in liver enzymes (ALT and AST) that were not significantly different from controls (*Online Supplementary Table S3, Online Supplementary Figures S7D-F, 8D-F*). Importantly, no deaths occurred in any treatment group during the 4-week study, and no weight loss, clinical signs, or overt toxicity were observed. Collectively, these results demonstrate that the GSK+VEN





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Figure 4. Combination of GSK269962A and venetoclax reduces the leukemia burden in acute myeloid leukemia patient-derived xenograft model. (A) Schematic showing the generation of luc-labeled patient-derived xenograft (PDX) mouse model using primary acute myeloid leukemia (AML) patient 1265 cells. These primary patient cells were transduced overnight with a lentiviral vector expressing EGFP and luciferase (LUC2) genes. Unsorted cells (p0-passage 0) after 24 hours of transduction were injected into NRG-S mice. GFP-sorted bone marrow (BM) cells harvested from primary PDX (P1-passage 1) mice on day 147 post transplant were cultured *ex vivo* for 12 days prior to injection into secondary recipients (P2-passage 2). (B) The BM cells from secondary recipient mice (P2) were injected intravenously into NRG-S mice at 0.6 million/mice (P3-passage 3). The mice were randomized into four experimental groups (Vehicle, GSK269962A [GSK], venetoclax [VEN], and GSK+VEN combination) of 5–7 mice each based on bioluminescence imaging (BLI) signals after day 13 post transplant. The mice were treated with GSK (30 mg/kg, p.o., Mon–Sat), VEN (50 mg/kg, p.o., Mon–Sat), and GSK+VEN combination for three weeks and monitored for leukemia burden through regular *in vivo* imaging. (C) The BLI signals were quantified, and leukemia burden was represented in terms of average radiance (p/s/cm²/sr). (D) Flow analysis of BM cells from 1265-luc PDX mice quantified engraftment of human CD45 positive cells or GFP positive cells. Data are presented as mean $\pm$ standard error of mean (SEM) (N=3–6) and analyzed by 2-way ANOVA (Tukey's multiple comparisons test). *** P <0.005 and **** P <0.0001 indicate statistical significance compared to vehicle control. (E) Representative density plots showing the relative percent populations of hCD45 and EGFP positive cells in the BM cells collected from the experimental PDX mice groups.

combination is well tolerated with both short-term and extended repeated dosing.

GSK269962A treatment potentiates the venetoclax-induced mitochondrial depolarization, reactive oxygen species levels, and apoptosis in acute myeloid leukemia cells

To test the effect of GSK on VEN-induced apoptosis, Annexin V/PI staining and caspase-3/7 activation were measured in both the parental and VEN-resistant MV4-11 cells. As expected, treatment with VEN or GSK alone exhibited dose-dependent caspase-3/7 activation and an increase in Annexin V-positive cells in both naïve and VENR cells (Figure 5A–D). The GSK+VEN combination resulted in significantly enhanced activation of Caspase-3/7 and Annexin V-positive cells in MV4-11 (Figure 5A, C) and MV4-11/VENR cells (Figure 5B, D) compared to VEN treatment. As VEN is known to inhibit mitochondrial respiration and alter mitochondrial outer membrane permeabilization (MOMP),²⁵ we investigated the effect of GSK co-treatment on VEN-induced $\Delta\Psi_m$ and the associated cellular ROS production. Both GSK and VEN, when used as single agents, exhibited a dose-dependent increase in mitochondrial depolarization and ROS in MV4-11 and MV4-11/VENR cells (Figure 5E–H). Interestingly, the GSK+VEN combination resulted in a significant increase in depolarized and ROS-positive MV4-11 (Figures 5E, G) and MV4-11/VENR (Figure 5F, H) cells compared to VEN treatment. These results indicate that GSK potentiates VEN-induced MOMP, thereby elevating ROS levels in AML cells to induce synergistic apoptosis. Although VEN inhibits mitochondrial respiration, AML cells have sustained $\Delta\Psi_m$ through ATP hydrolysis and developed resistance to therapy.²⁶ To measure $\Delta\Psi_m$ in intact cells, we measured TMRM (tetramethylrhodamine methyl ester) fluorescence after 15 hours (h) and 24 h of treatment. MV4-11 cells showed active voltage across the membrane with an increase in TMRM fluorescence (hyperpolarization) after 15 h of treatment (single and combination), whereas 24 h of treatment with GSK and GSK+VEN combination resulted in decreased TMRM fluorescence (depolarization) (Figure 5 I). Similarly,

MV4-11/VENR cells exhibited a biphasic response (hyperpolarization at 15 h followed by normalization at 24 h) with GSK treatment (Figure 5J). In line with our TMRM findings, the intact MV4-11/VENR cells treated with GSK exhibited higher maximal rate of respiration (Figure 5K). The oxidative phosphorylation (OXPHOS) capacity in permeabilized MV4-11/VENR cells indicated that GSK targets Complex IV (Figure 5L). The parental MV4-11 cells only exhibited synergistic effect on TMRM fluorescence (mitochondrial polarization) in viable intact cells very similar to that of activity seen in bulk cells (Figure 5G) with GSK+VEN combination. The effects of GSK+VEN combination on mitochondrial polarization were suboptimal in both bulk (Figure 5H) and viable intact VENR cells (Figure 5J). These findings suggest that the combined effect of GSK and VEN is mediated by direct action on mitochondria that alter MOMP and increase ROS, ultimately leading to synergistic cytotoxicity in AML cells. Altogether, GSK+VEN combination effectively induces apoptosis through mitochondrial depolarization and elevated ROS levels in naïve and VEN-resistant AML cells.

GSK269962A treatment suppresses venetoclax-induced pro-survival proteins to promote apoptosis in acute myeloid leukemia cells

To investigate the mechanism of synergy between GSK and VEN in promoting apoptosis, we performed immunoblotting analyses in MV4-11 and MV4-11/VENR cells following treatment with GSK, VEN, or GSK+VEN combination. The levels of cleaved CASPASE-3 were dramatically increased with the combination treatment, consistent with a synergistic effect of GSK and VEN in inducing AML cell death (Figure 6A). Additionally, the steadily increased levels of cleaved PARP-1 (poly(ADP-ribose) polymerase-1), a known substrate for caspases, with combination treatment compared to single agents, suggest effective induction of apoptosis in both VEN-sensitive and VEN-resistant cells. The level of ROCK1 decreased with GSK treatment, consistent with the previous report,¹⁹ and there was an increase in the shorter isoform of ROCK1 with the combination treatment. The level of ROCK2 appeared to be stable with a subtle

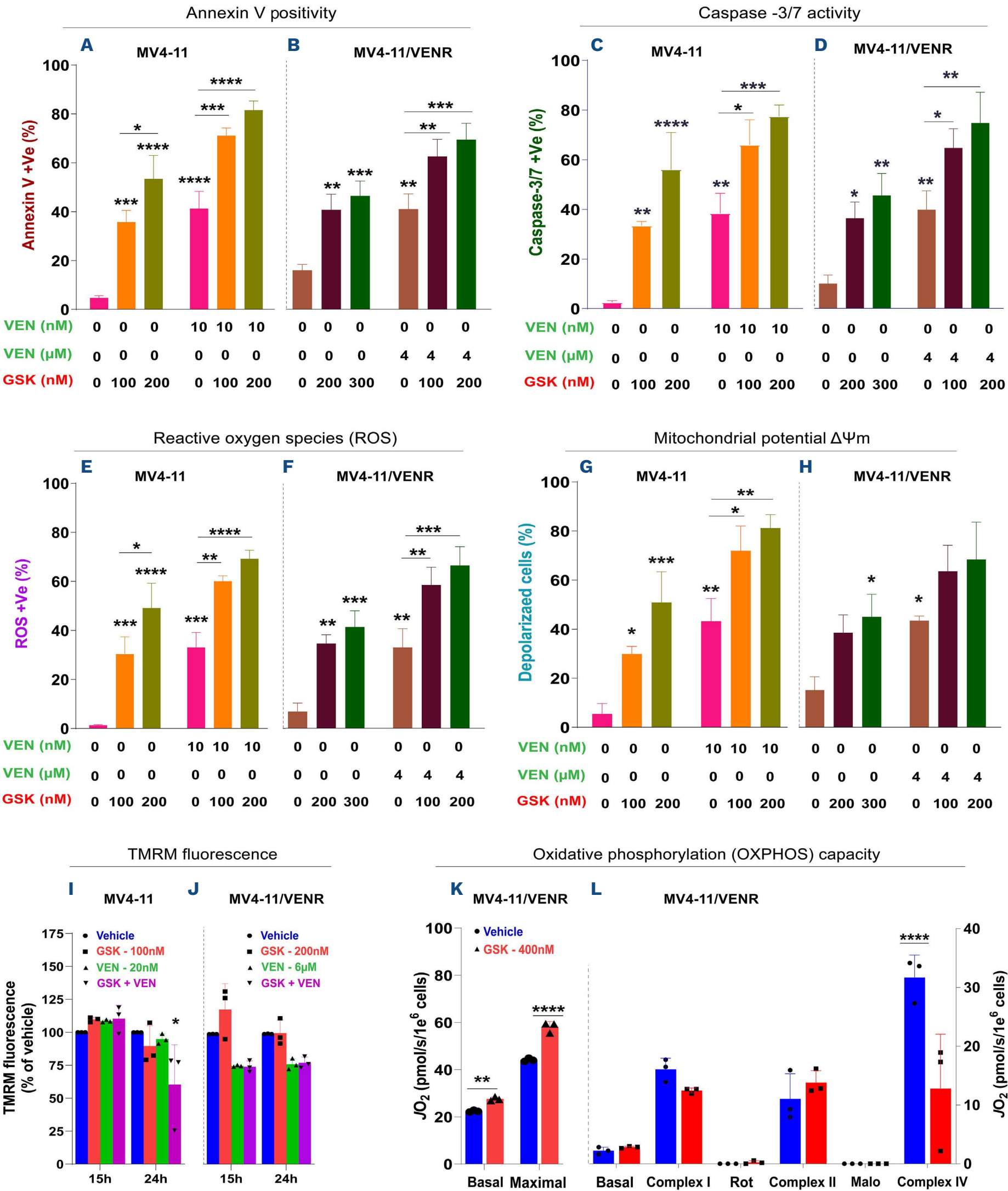


Figure 5. GSK269962A potentiates venetoclax-induced apoptosis, reactive oxygen species and mitochondrial membrane depolarization in acute myeloid leukemia cells. Measurement of (A and B) Annexin V levels, (C and D) caspase-3/7 activity, (E and F) reactive oxygen species (ROS) levels, and (G and H) mitochondrial membrane depolarization levels in MV4-11 and MV4-11/VENR cells after 48 hours (h) of treatment with Vehicle, GSK269962A (GSK), venetoclax (VEN), and GSK+VEN combination. Data are presented as mean ± standard deviation (SD) (N=3) and analyzed by 2-way ANOVA (Tukey's multiple comparisons test). (I and J)

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Flow cytometric analysis of intact cell $\Delta\Psi_m$ in (I) MV4-11 and (J) MV4-11/VENR cell lines after 15 and 24 h of treatment. Data expressed as a percentage of maximal TMRM fluorescence relative to vehicle. Data are presented as mean $\pm$ standard error of mean (SEM) (N=3) and analyzed by 2-way ANOVA (Dunnett's multiple comparisons test). (K and L) Measurement of OXPHOS capacity in intact (K) and permeabilized cells after 14 h of treatment. Data are presented as mean $\pm$ SEM (N=3) and analyzed by 2-way ANOVA (Sidak's multiple comparisons test). * P <0.05, ** P <0.01, *** P <0.005, **** P <0.0001.

decrease in the phosphorylated PTEN (phosphatase and tensin homolog) levels after the treatments.

As ROCK1 is known to undergo CASPASE-3-mediated cleavage,²⁷ the appearance of constitutively active cleaved-ROCK1 correlate with higher cellular apoptosis induced by combination treatment compared to single agents. As expected, treatment with ROCKi GSK resulted in lower levels of phosphorylated COFILIN, a downstream protein regulated by ROCK activation. Intriguingly, AML cells treated with VEN showed increased levels of phosphorylated COFILIN, which mimics the activation of ROCK1 independent of Rho activity via CASPASE-3-directed truncation.²⁷ However, the addition of GSK suppressed the VEN-mediated ROCK1 activation as indicated by decreased levels of phospho-COFILIN (the inactive form). The activation of COFILIN through dephosphorylation has been shown to result in its mitochondrial translocation, initiating intrinsic apoptosis by opening the mitochondrial permeability transition pore and subsequent release of cytochrome c.^{28,29} Thus, the COFILIN activation with GSK+VEN combination might be a crucial mechanism for the induction of mitochondrial apoptosis in both naïve and VEN-resistant AML cells.

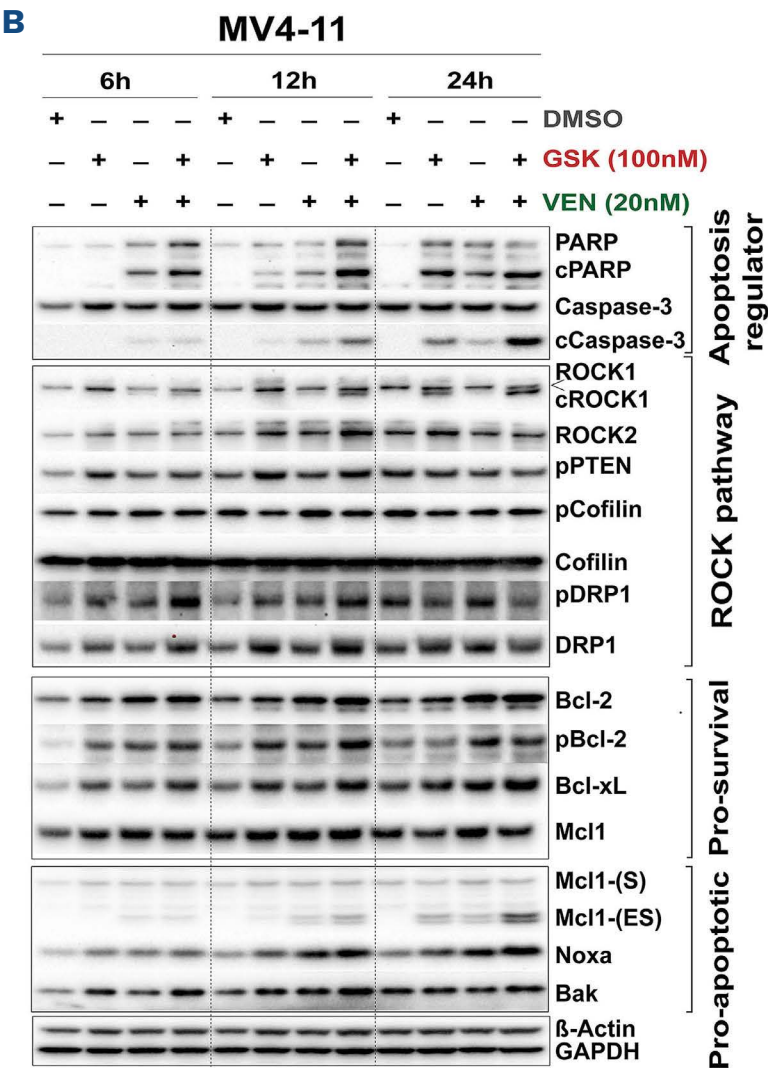
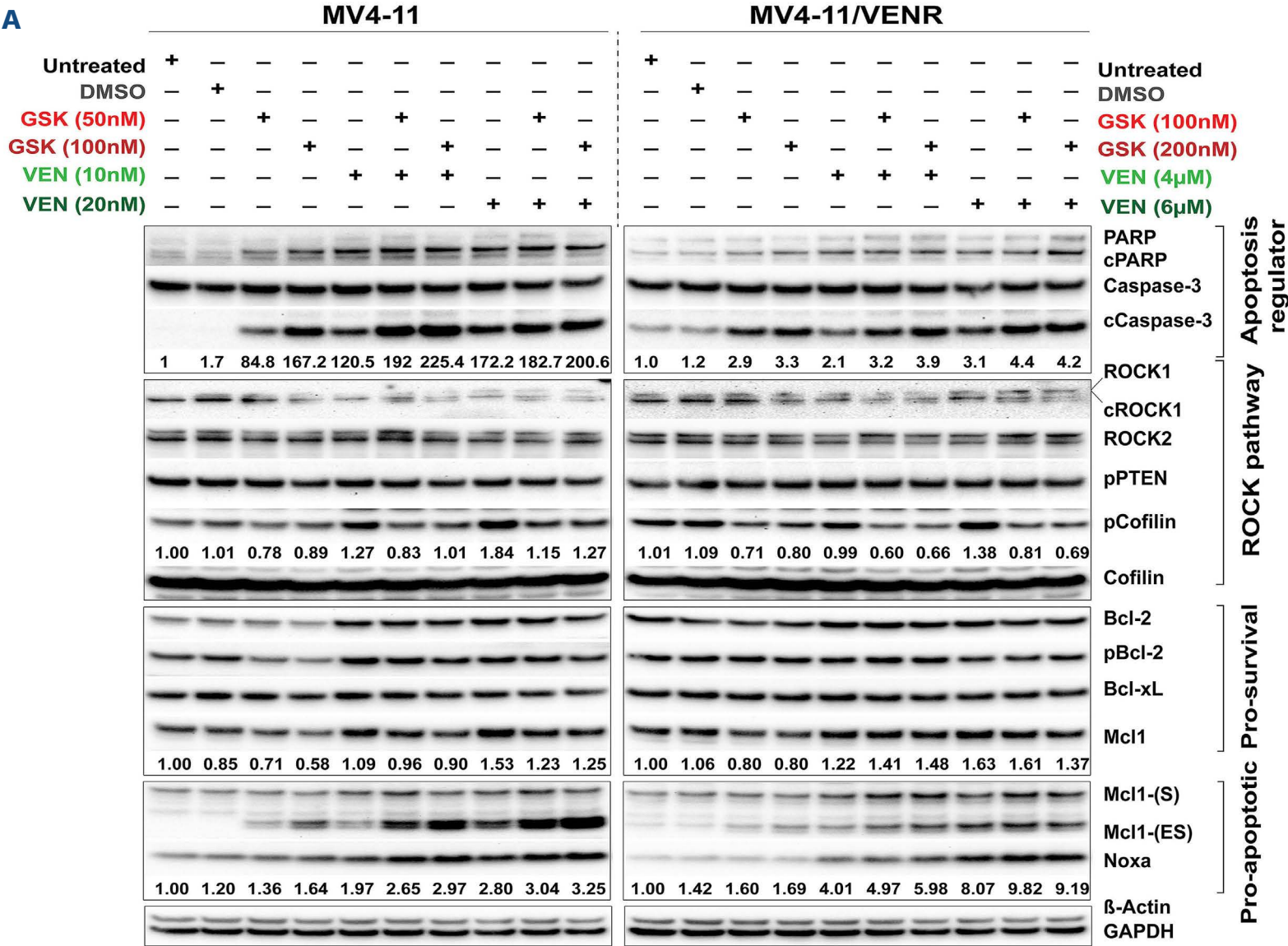
Next, immunoblotting analysis of different BCL-2 family proteins showed that the addition of GSK suppressed the levels of phosphorylated BCL-2, a pro-survival protein induced by VEN in MV4-11 cells (Figure 6A). Moreover, the VEN-induced MCL1 (anti-apoptotic protein associated with VEN resistance) levels were suppressed by GSK addition in AML cells. The pro-apoptotic proteins such as NOXA and shorter isoforms of MCL1 (short [S] and extra short [ES])^{30,31} were increased with GSK+VEN combination compared to single-agent treatment and correlated with higher levels of caspase activity and apoptosis (Figure 6A). The upregulation of NOXA, a pro-apoptotic BCL-2 member that neutralizes MCL1 and other anti-apoptotic BCL-2 family proteins,³² and the suppression of VEN-induced MCL1 levels by GSK imply a pro-apoptotic mechanism that contributes to synergistic cytotoxicity of GSK+VEN combination (Figure 6A). These effects were seen consistently in both the naïve and VEN-resistant MV4-11 AML cells.

Next, we tested the early dynamics of target protein expression by immunoblotting analysis. Interestingly, GSK+VEN combination resulted in the appearance of cleaved-PARP (apoptosis mediator) and executionary cleaved CASPASE-3 as early as 6 h, with a gradual increase over 12 h and 24 h of treatment compared to single agents (Figure 6B). Accordingly, the cleaved ROCK1 levels were higher after 12 h and 24 h of GSK+VEN treatment compared to single agents alone. The cells treated with GSK+VEN exhibited

upregulation of ROCK1/2, Dynamin-related protein 1 (DRP1), phosphorylated DRP1 (activation) and PTEN after 6 h and 12 h of treatment, with normalization after 24 h of treatment. The increased levels of pro-apoptotic BAK after 6 h and 12 h of GSK+VEN treatment relative to single agents were consistent with synergistic apoptosis induction. Pro-apoptotic proteins, including NOXA and MCL1-ES, were also strongly up-regulated following GSK+VEN combination compared to either agent alone. Although pro-survival BCL-2 members (BCL-2 and BCL-XL) were up-regulated, MCL1 levels were suppressed after 24 h of treatment with GSK and the GSK+VEN combination (Figure 6B). Overall, time-course immunoblotting analysis demonstrated that the GSK+VEN combination increases the expression of multiple apoptosis regulators and primes AML cells for cell death in a time-dependent manner. These findings indicate that GSK antagonizes pro-survival proteins induced by VEN, while increasing pro-apoptotic proteins to synergistically induce cell death in both VEN-sensitive and VEN-resistant AML cells (Figure 6C).

Discussion

In this study, we established a synergistic antileukemic interaction between BCL-2 and ROCK inhibitors in multiple preclinical AML models. VEN, a clinically approved and orally bioavailable BCL-2 inhibitor with a well-defined pharmacokinetic profile, provided a robust backbone for exploring such combinations, whereas GSK represents a newer, preclinical, orally available ROCK inhibitor. In AML pathogenesis, aberrant ROCK signaling has been linked to leukemic cell survival, drug resistance, and leukemic stem cell maintenance.^{16,33} ROCKi has proven effective in AML and various solid tumors, highlighting the pathway's broader therapeutic significance.³⁴ All three structurally different ROCKi (DJ4, Fasudil, GSK) synergized with VEN in triggering cell death in AML cells *in vitro*. Nonetheless, while exhibiting *in vitro* synergistic action in conjunction with VEN, the preliminary *in vivo* trials of DJ4 and Fasudil co-administration with VEN revealed only moderate activity, attributable to dose-limiting toxicities and adverse pharmacokinetic properties. We, therefore, focused on GSK, an orally bioavailable ROCKi that has enhanced selectivity and tolerability. GSK demonstrated significant anti-leukemic activity *in vivo* when administered in conjunction with VEN in multiple CDX models. All the tested primary AML patient cases with diverse genetic subtypes exhibited additive to synergistic cytotoxicity with GSK+VEN combination *ex vivo*.



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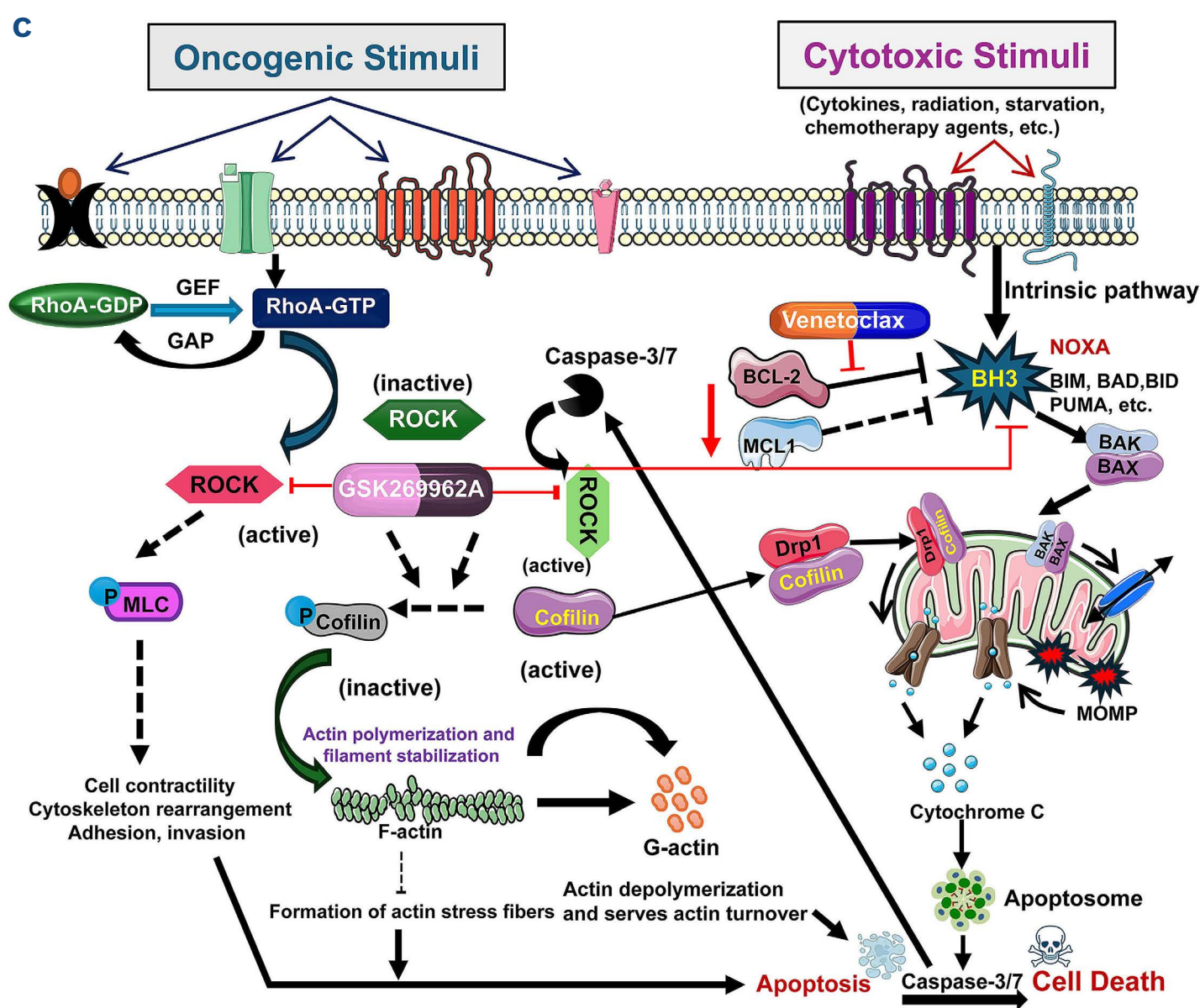


Figure 6. GSK269962A blocks venetoclax-driven pro-survival signaling and promotes apoptosis in AML cells. (A) Immunoblotting analysis of MV4-11 and MV4-11/VENR cells after 48 hours (h) of treatment with vehicle (DMSO-D) indicated doses of GSK269962A (GSK), venetoclax (VEN), and GSK+VEN combination. The levels of phospho-Cofilin (pCofilin), Mcl1 (anti-apoptotic), Noxa (pro-apoptotic), and cleaved caspase-3 (cCaspase-3) were quantified relative to GAPDH and normalized to untreated control cells. Mcl1 (S) and Mcl1 (ES) represent the “short” (S) and “extra short” (ES) isoforms that exhibit pro-apoptotic activity. GAPDH and β -Actin levels were used as loading controls. Representative blots from 3 independent experiments are shown. (B) Immunoblotting analysis of MV4-11 cells after 6, 12, and 24 h of treatment either with DMSO, GSK-100 nM, VEN-20 nM, or GSK+VEN combination. GAPDH and β -Actin levels were used as loading controls. Representative blots from 2 replicates are shown. (C) Proposed mechanisms of action of GSK in combination with VEN. Inhibition of rho-kinases (ROCK1/2) with GSK increases active cofilin levels, leading to actin depolymerization and F-actin destabilization, which promote apoptotic pathways and impair cell contractility, adhesion, and invasion. Active cofilin (dephosphorylated) is known to translocate to mitochondria in complex with Drp1, where it promotes intrinsic apoptosis through mitochondrial outer membrane permeabilization (MOMP). Inhibition of anti-apoptotic Bcl-2 with VEN increases the activity of pro-apoptotic BH-3 only proteins like Noxa and facilitates Bax or Bak oligomerization, triggering MOMP and apoptosis. Treatment with GSK results in downregulation of anti-apoptotic proteins (BCL-2, MCL1) and upregulation of Noxa, further enhancing cell susceptibility to VEN. In addition, GSK inhibits ROCK activation induced by VEN via caspase-mediated cleavage, leading to accumulation of apoptotic signals and more cell death. The artworks were adopted from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Commons Attribution 4.0 Unported License.

GSK+VEN combination showed a significant efficacy in PDX mouse model generated from primary AML patient cells. This novel combination was found to be safe in normal mice after four weeks of treatment with no significant changes in renal function, liver function, electrolytes, and albumin levels. Regarding the CBC, mild to moderate reduction in white blood cell counts was observed in the GSK+VEN combination group, particularly in neutrophils, lymphocytes, and monocytes, a common pattern seen with many clinically active anti-leukemia agents. Importantly,

these changes remained within normal limits, and there were no clinical signs of infection during or after the treatment. Hemoglobin and platelet counts did not show any clinically or statistically meaningful changes. No deaths or treatment-related toxicity were observed across four CDX models treated for up to nearly four weeks or in two dedicated independent 2- and 4-week safety studies. The late deaths of 2 PDX animals treated with GSK may not have been due to the neoplasm, and testing long-term toxicity of GSK+VEN combination is of interest prior to fu-

ture clinical trials; however, this is beyond the immediate scope of this work.

Mechanistically, GSK triggered apoptosis characterized by caspase-3/7 activation and markedly increased VEN-induced cytotoxicity. Our findings suggest that mitochondrial depolarization and increased ROS production may contribute to the synergistic cytotoxicity of GSK and VEN in both naïve and VEN-resistant AML cells. AML cells demonstrate inherent deficiencies in OXPHOS, and the restoration of oxidative ATP generation (hyperpolarization) has proven to be significantly lethal.³⁵ We observed early mitochondrial hyperpolarization (TMRM fluorescence at 15 h) and depolarization at 24 h, suggesting a priming effect that enhances the susceptibility of leukemic cells to apoptosis. Many chemotherapeutics, including VEN, have been shown to disrupt respiration in AML cells.^{36,37} VEN, a known OXPHOS inhibitor, has been shown to inhibit proton-pumping mitochondrial respiratory complexes (I/II/IV) to disrupt respiratory flux in AML cells.³⁷ Interestingly, GSK showed inhibitory activity on complex IV in VEN-resistant MV4-11 cells, contradicting higher basal and maximal respiration levels observed with GSK treatment. Recently, it has been demonstrated that ROCK kinases mediate metabolic adaptation of cancer cells, and co-inhibition of ROCK and OXPHOS resulted in synergistic anticancer activity.³⁸ Therefore, our findings support the existing literature and suggest further mechanistic studies to explore how GSK-mediated biphasic effects on respiration align with VEN activity to induce apoptosis in AML cells.

The coordinated activity of BCL-2 family proteins is crucial for determining cell fate and is the key regulator of mitochondrial apoptosis.³⁹ The clinical utility of VEN, a selective BCL-2 inhibitor, is limited due to adaptation of cells and development of resistance resulting from the overexpression of other antiapoptotic BCL-2 family members such as MCL1 and BCL-XL.^{40,41} Simultaneous targeting of BCL-2, BCL-XL, and MCL1 has been shown to exhibit synergistic cytotoxicity and rescue resistance to VEN.^{42,43} As a single agent, GSK has been shown to down-regulate the expression of anti-apoptotic proteins, including Survivin, MCL1, and BCL-XL, in AML cells.¹⁹ Accordingly, the addition of GSK suppressed VEN-induced overexpression of BCL-2 and MCL1, while the effect on BCL-XL was subtle. Additionally, we observed a significant upregulation of pro-apoptotic MCL1-S/ES isoforms and NOXA proteins with GSK+VEN combination in naïve and VEN-resistant AML cells. These shorter spliced variants of MCL1 are known to interact with unspliced MCL1 and promote mitochondrial apoptosis.^{30,44} MCL1-ES is a distinct BCL-2 family protein that induces mitochondrial apoptosis independent of BAX and BAK, whereas other conventional BCL-2 family physiology is dependent on BAX and/or BAK oligomerization to promote apoptosis.³¹ Therefore, we assume that the GSK+VEN combination elicits a synergistic induction of apoptosis through the distinctive activation of interactions

between BCL-2 members and primed VEN-resistant cells susceptible to VEN. It has been previously shown that the levels of NOXA, a BH3-only pro-death BCL-2 member that inhibits MCL1, determine the sensitivity to BH3 mimetics and apoptosis.^{45,46} Consistent with other VEN-based combinations,^{47,48} the synergy between GSK and VEN in AML cells may be attributed to the induction of NOXA levels. Binding of NOXA to MCL1 was known to induce proteasome-mediated degradation of MCL1.⁴⁹ We observe a negative correlation between NOXA and MCL1 levels to some extent following GSK+VEN combination. In parallel, GSK inhibited VEN-induced phospho-Cofilin (inactive) levels and thus might be facilitating translocation of active Cofilin to mitochondria and interact with DRP1 to promote MOMP and apoptosis.^{28,29} Altogether, our data suggest that the GSK+VEN combination targets distinct mechanisms to promote synergistic apoptosis in AML cells. Though beyond the scope of this work, our findings support future mechanistic studies to unfold complex interactions between BCL-2 family members and other apoptosis mediators resulting from GSK+VEN combination.

While the evaluated ROCKi agents are not yet ready for clinical use, these results establish a foundation for future advances. Fasudil and DJ4 require intraperitoneal administration and exhibit suboptimal pharmacokinetic profiles, underscoring the need for reformulation to improve their bioavailability and overall clinical applicability. GSK is an orally bioavailable compound, yet its long-term safety profile still has to be fully defined. In spite of these observations, our findings indicate that ROCK inhibition may enhance the effectiveness of VEN against leukemia while remaining tolerable, warranting further preclinical studies with the potential to advance to early-phase clinical trials.

Disclosures

DJF has received research funding, honoraria, and/or stock options from AstraZeneca, Dren Bio, Recludix Pharma, and Kymera Therapeutics. TPL has Scientific Advisory Board membership; and has received consultancy fees, honoraria, and/or stock options from Keystone Nano, Flagship Labs 86, Dren Bio, Recludix Pharma, Kymera Therapeutics, and Prime Genomics. RLL is a scientific advisor to Imago, Mission Bio, Zentalis, Ajax, Auron, Prelude, C4 Therapeutics, and Isoplexis; receives research support from Ajax, Zentalis, and Abbvie; has consulted for Incyte, Janssen, and Astra Zeneca; and has received honoraria from Astra Zeneca for invited lectures. All the other authors have no conflicts of interest to disclose.

Contributions

UG conceptualized the study design, was responsible for the experimental work, data collection and analysis, wrote the first draft, and revised the manuscript. AS conceptualized the study design, was responsible for the experimental work, data collection and analysis, provided project oversight, was responsible for the funding acquisition, helped write

the first draft, and revised the manuscript. DFC conceptualized the study design, provided project oversight, helped write the first draft, and was responsible for the funding acquisition. TPL provided scientific resources and subject matter expertise, and was responsible for the funding acquisition. RB, CA, SP, VSB, SFT and KFW were responsible for the experimental work, and data collection and analysis. MCC, DJF, KFW, JH, GLS, DD, RLL, SM, HZ, KM and SD provided scientific resources and subject matter expertise. VSB revised the manuscript. All authors edited, reviewed, and approved the final manuscript for publication.

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Data-sharing statement

The data supporting the findings of this study are available within the article and/or its Online Supplementary Appendix. Additional information is available upon request from the corresponding author.

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