SUMOylation inhibitor TAK-981 (subasumstat) synergizes with 5-azacytidine in preclinical models of acute myeloid leukemia

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Supplementary Methods

Generation of bioluminescent AML cell lines

THP-1-LucZsGreen and U937-LucZsGreen cell lines were obtained by retroviral gene transfer. Retroviruses were produced by cotransfection of HEK293T cells with the pHIV-Luc-ZsGreen (Addgene, Plasmid #39196), psPAX2 (Addgene, Plasmid #12260) and pCMV-VSV-G (Addgene, Plasmid #8454) vectors using Lipofectamine 2000 (Invitrogen). Viral supernatants were collected 48 hours after transfection, 0.45 µm-filtered, and used to infect AML cell lines. Two weeks after infection, ZsGreen-positive cells were cloned using a FACSAria cell sorter (Becton Dickinson).

Patient cells culture

Immediately after collection, fresh leukocytes were purified by density-based centrifugation using Histopaque 1077 (Sigma-Aldrich), frozen and stored in liquid nitrogen. After thawing, leukocytes were cultured in the StemSpan SFEM II culture medium (StemCell Technologies), supplemented with StemSpan CD34+ expansion supplement and UM729 according to supplier's recommendations. Cells were collected and analyzed after 8 days of culture.

IC_{50} measurement and synergy matrices

For 24 hours IC_{50} measurements, medium was complemented with drugs and analyzed 24 hours later. For 72 hours IC_{50} measurements, cells were treated on day 1 and 2. Cells were diluted twice on day 3 and treated with drugs and cell viability was analyzed 24 hours later. Cell viability was measured using the MTS viability assay (Promega) according to supplier's protocol. For PBMC, which metabolize MTS poorly, cell viability was measured by flow cytometry using FSC/SSC gating to select living cells. Absolute IC_{50} were calculated using the GraphPad PRISM software (version 9). Zero-interaction potency (ZIP) score gives a measure of drug interaction relationship by comparing the change in the potency of the dose-response curves between individual drugs and their combinations. ZIP score was calculated using online SynergyFinder software v2.0 (https://synergyfinder.fimm.fi)[1].

Immunoblots

Equal number of cells were collected and directly lysed in Laemmli electrophoresis sample buffer. Antibodies against SUMO-1 (21C7), and SUMO-2/3 (8A2) were obtained from the Developmental Studies Hybridoma Bank.

Flow cytometry

Cells were washed in PBS containing 5% FBS, incubated at 4°C for 30 minutes with conjugated antibodies (see Table 1), washed with PBS and analyzed by flow cytometry with a Novocyte flow cytometer (Agilent). Median fluorescence intensities (MFI) or the percentages of positive cells were calculated using the NovoExpress software (v.1.5.6). For the experiments with mice, bone marrows (from tibias and femurs) and spleens were dissociated and cells were rinsed in PBS. After red blood cells lysis using the ACK lysis buffer (A1049201, Gibco), mononuclear cells from bone marrow and spleen were labeled as described above. Anti-hCD45 antibodies were used to identify human blasts as CD45 is expressed on human leukocytes. For cell cycle analysis, cells were washed once in PBS and fixed with 70% ethanol at -20°C for 30 minutes. Cells were then washed and resuspended in PBS-0.1% Triton complemented with 100 µg/mL RNAse A (Sigma) and 5 µg/mL propidium iodide (BD, 51-66211E) at 37°C for 30 minutes. After PBS washing, cellular DNA contents were assayed by flow cytometry.

Target	*Fluorochrome*	*Manufacturer*	*Reference*
CD3	FITC	Miltenyi	130-113-138
	VioBlue	Miltenyi	130-110-460
CD56	APC-Vio770	Miltenyi	130-114-548
	FITC	Miltenyi	130-100-683
CD69	PE	Miltenyi	130-112-651
CD14	APC-Vio770	Miltenyi	130-110-552
	PE	Miltenyi	130-110-519
CD45	FITC	Miltenyi	130-110-633
	APC	Miltenyi	130-113-114
CD15	PE-Vio770	Miltenyi	130-113-486
IFNa	PE	Miltenyi	130-116-873
ICAM-1	APC	Miltenyi	130-121-342
MICA/B	PE	Miltenyi	130-118-829
Annexin-V	FITC	Miltenyi	130-093-060
7AAD	-	ThermoFisher	00-6993-50

Supplementary Table 1: Antibodies used for Flow Cytometry

In vivo Bioluminescence Imaging

The engraftment of AML bioluminescent cell lines (THP-1-LucZsGreen and U937-LucZsGreen) was assessed by bioluminescence imaging using an IVIS Spectrum In Vivo Imaging System (Perkin Elmer). Mice were intraperitoneally injected with 3 mg of D-luciferin resuspended in 0.9% NaCl, 20 min before imaging and were then anesthetized using 2.5% Isoflurane. Mean of total body bioluminescent signal quantification (photons/ROI/min) of regions of interest was carried out using Living Image software.

In vivo treatments

Once engrafted, mice were assigned to the different treatment arms based on tumor burden and body weight. AZA (2 mg/kg) was administered by intra-peritoneal injection, TAK-981 (15 mg/kg) by caudal tail vein injection and venetoclax (50 mg/kg) by oral gavage. Immediately before administration, AZA was solubilized in 0.9% NaCl, TAK-981 in 20% HPBCD (hydroxypropyl beta-cyclodextrin) and venetoclax in corn oil (SIGMA, C8267) with 30% PEG400 and 10% ethanol. Evolutions of tumor burden were monitored by bioluminescence (cell lines) or by flow cytometry (hCD45+ cells) in the peripheral blood (primary AML cells). Mice were monitored daily for symptoms of distress defined by the Ethical Committee (ruffled coat, hunched back, and reduced mobility) to decide the time of killing of injected animals.

Analysis of SUMOylation activity in bone marrow cells

Bone marrows (from tibias and femurs) were flushed and cells were rinsed in PBS. After red blood cells lysis using the ACK lysis buffer (A1049201, Gibco), mononuclear cells from bone marrow were counted and equal number of cells were used to prepare extracts and monitor SUMOylation activity as previously described[2]. Briefly, 2.10^6 cells are resuspended in 50 µL of swelling buffer (20 mM Hepes pH 7.5, 1.5 mM MgCl2, 5 mM KCl, 1mM DTT, 1 µg/mL aprotinin, pepstatin, leupeptin), incubated on ice vortexing every 5 min for 30 min, lysed with 4 freeze/thaw cycles and passed through an hamilton seringe to shear DNA. After centrifugation, extracts are supplemented with 0.5µM SUMO-vinyl sulfones (Boston Biochem) and mixed and incubated for 45 min at 30 °C with XMap Luminex beads coupled to the ZMYM-5 protein, 2 mM ATP, 10 µM SUMO-1 in 20 mM Hepes pH 7.3, 110 mM KOAc, 2 mM Mg(OAc)2, 0.05% Tween-20, 0.5 mM EGTA, 0.2 mg/mL ovalbumin, 1 mM DTT, 1 µg/mL aprotinin, pepstatin, leupeptin. After washes with PBS, 0.05% Tween-20, 0.5% SDS, beads are incubated with mouse anti-SUMO-1 antibody (21C7) for 1 hr, washed with PBS, 0.05% Tween-20 and

incubated with Alexa-Fluor 488-coupled secondary antibody for 30 min before analysis by flow cytometry[2].

RNA-seq libraries preparation and sequencing

Total RNAs were purified using the GenElute Mammalian Total RNA kit (Sigma-Aldrich), treated with DNase I (New England Biolabs) and re-purified. RNA quality was assessed using a BioAnalyzer Nano 6000 chip (Agilent). Three independent experiments were performed. Libraries were prepared using TruSeq®Stranded mRNA Sample Preparation kit (Illumina). After the PCR amplification step, PCR products were purified using AMPure XP beads (Agencourt Biosciences Corporation). The quality, size and concentration of cDNA libraries were checked using the Standard Sensitivity NGS kit Fragment Analyzer and qPCR (ROCHE Light Cycler 480). Libraries were sequenced using an Illumina Novaseq 6000 sequencer as paired-end 150 base reads. Replicates 1 and 2 were sequenced on the Montpellier Genomix facility (MGX) and replicate 3 on the CNAG platform (Center for Genomic Regulation, Barcelona, Spain). Image analysis and base calling were performed using the NovaSeq Control Software, Real-Time Analysis 3 (RTA) and bcl2fastq. The RNA-Seq sequencing data are available on Gene Expression Omnibus with accession number GSE212330 (token for reviewers: wzcveigafrkhrob)

RNA-seq mapping, quantification and differential analysis

RNA-seq reads were mapped on the Human reference genome (hg38, GRCh38p12) using TopHat2 (2.1.1)[3] based on the Bowtie2 (2.3.5.1) aligner[4]. Reads association with annotated gene regions was done using the HTseq-count tool v0.11.1[5]. Differential expression analysis was performed with DESeq2[6] using normalization by sequencing depth and parametric negative binomial law to estimate the data dispersion. Genes with a fold change ≥ 2 or ≤ 0.5 and an adjusted p-value < 0.05 were considered differentially expressed. Gene Set Enrichment Analyses were performed using https://www.gsea-msigdb.org/gsea/index.jsp (version 4.0.3)[7].

RT-qPCR assays

After DNase I treatment, 1 µg of total RNA purified as described above was used for cDNA synthesis using the Maxima First Strand cDNA kit (ThermoFisher Scientific). qPCR assays were conducted using Taq platinum (Invitrogen) and the LightCycler 480 device (Roche) with specific DNA primers (IDT, sequence available on request). Data were normalized to the mRNA levels of the *GAPDH* housekeeping gene.

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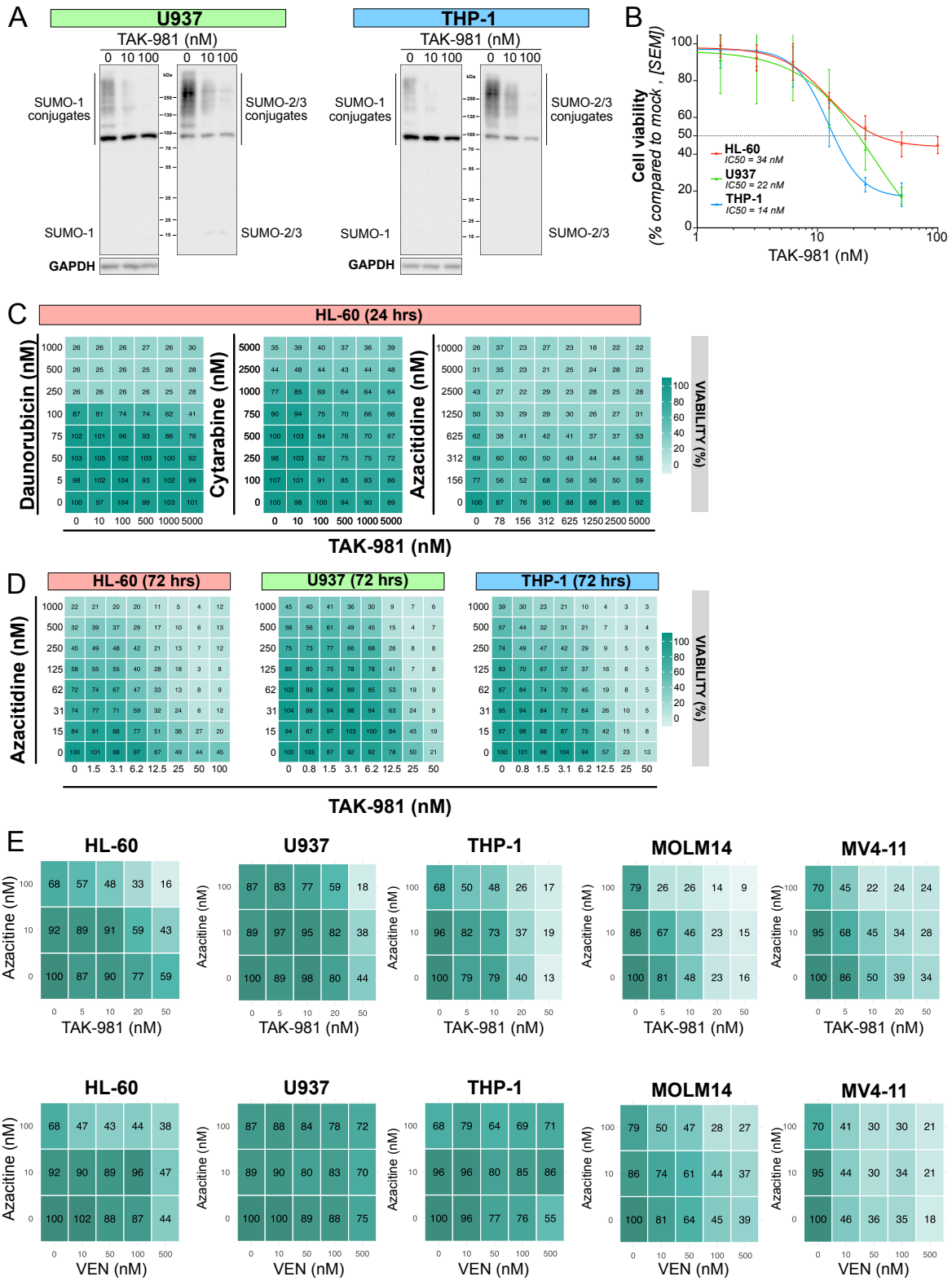
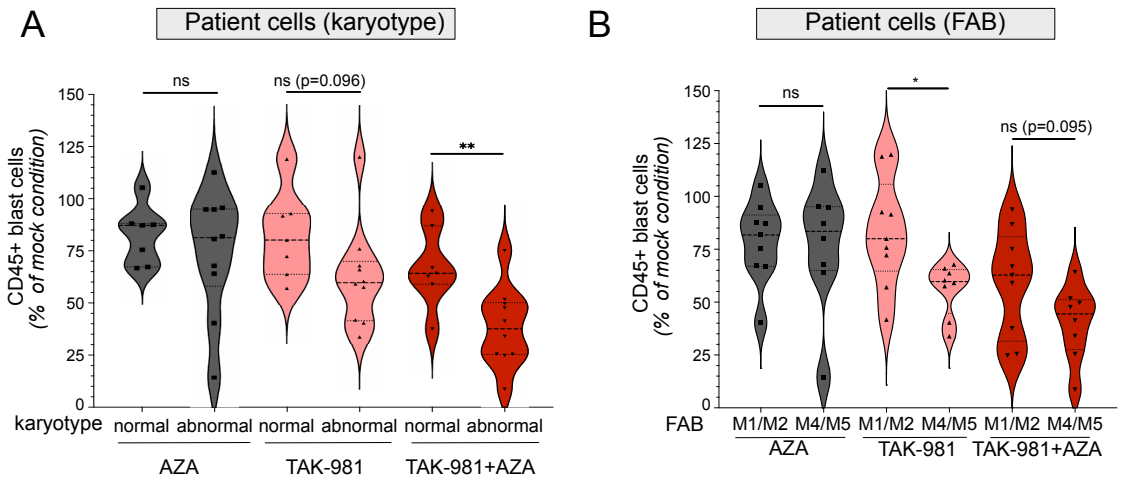
Supplementary Figures and Tables



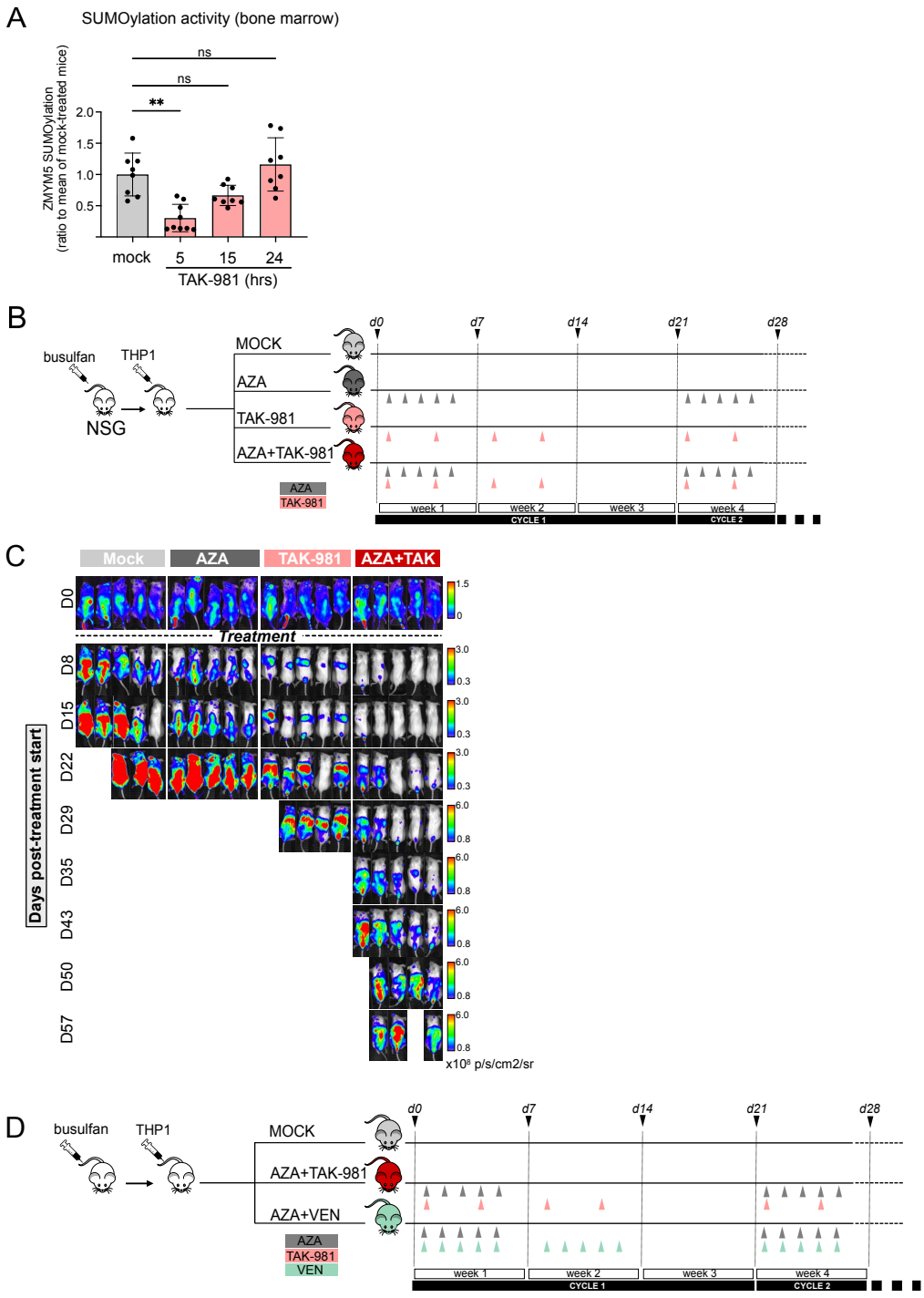
Supplementary Figure 1: TAK-981 induces deSUMOylation and synergize with Azacitidine to induce death of AML cell lines

A) U937 and THP-1 cells were treated with 10 nM or 100 nM of TAK-981 for 24 hours and immunoblots were performed for SUMO-1, SUMO-2/3 and GAPDH. **B)** IC_{50} determination of

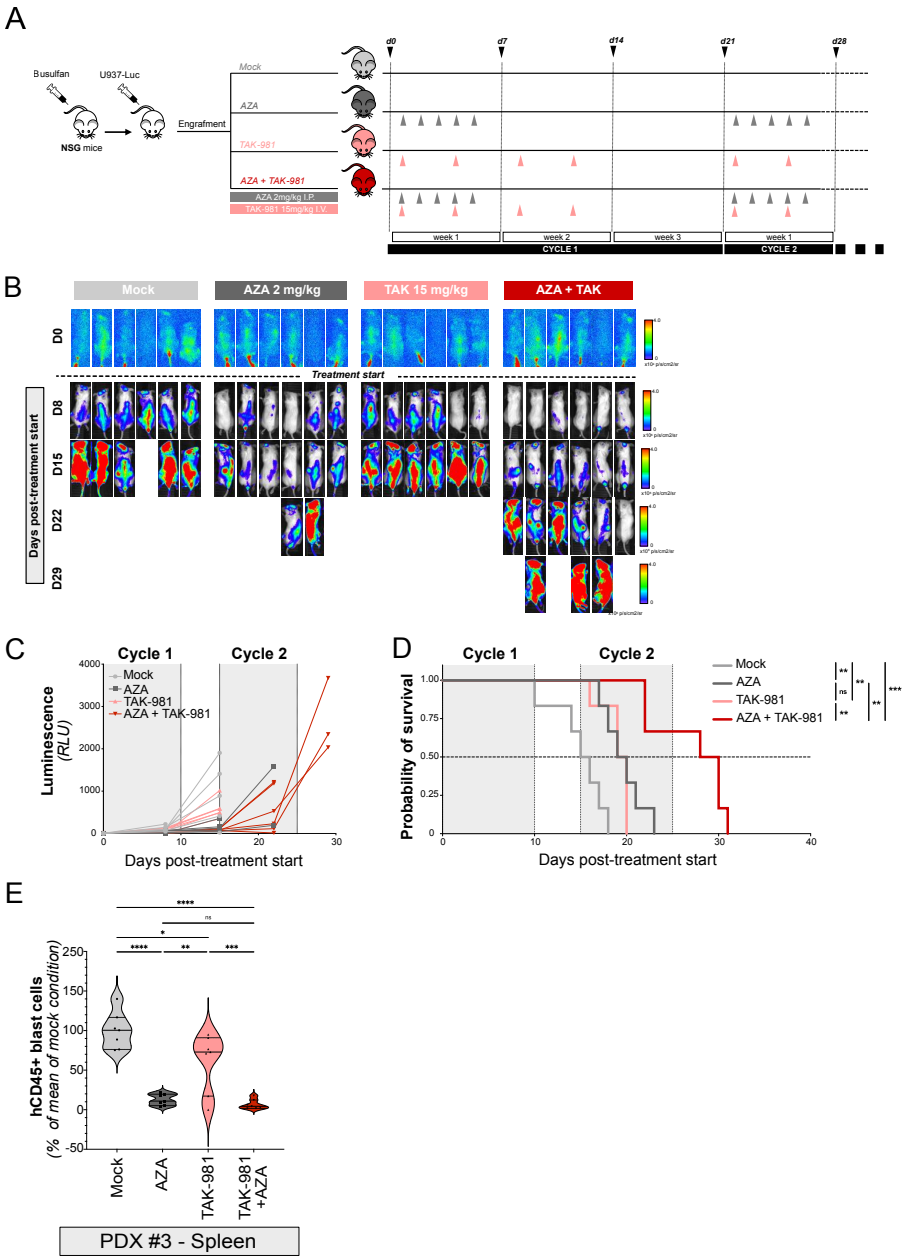
HL-60, U937 and THP-1 cell lines treated with TAK-981 at concentrations ranging from 1 to 100 nM for 72 consecutive hours. Cell viability was determined by MTS assays 24 hours after the last addition of drug and compared to that in mock-treated conditions. Concentration-response curves were generated comparing the viability in TAK-981 treated conditions with mock-treated controls (n=3, mean +/- SEM, absolute half-maximal inhibitory concentrations (IC_{50}) are shown). **C)** Heat maps showing the median percentage of viability of HL-60 cells treated for 24 hours with TAK-981 and either DNR, ARA-C or AZA compared to mock-treated conditions, assessed by MTS assay (median of 3 independent experiments for each drug). **D)** Heat maps showing median percentage of viability for HL-60, U937 and THP-1 cells treated with TAK-981 and AZA every day for 3 consecutive days. Viability was analyzed at day 4 by MTS and compared to that in mock-treated conditions (median of 3 independent experiments for each cell line). **E)** Heat maps showing median percentage of viability for HL-60, U937, THP-1, MOLM14 and MV4-11 cells treated with AZA (10 or 100 nM) combined to TAK-981 (5, 10, 20, 50 nM) or VEN (10, 50, 100, 500 nM) every day for 3 consecutive days. Cell viability was determined by MTS assays 24 hours after the last addition of drug and compared to that in mock-treated conditions (median of at least 3 independent experiments for each cell line).



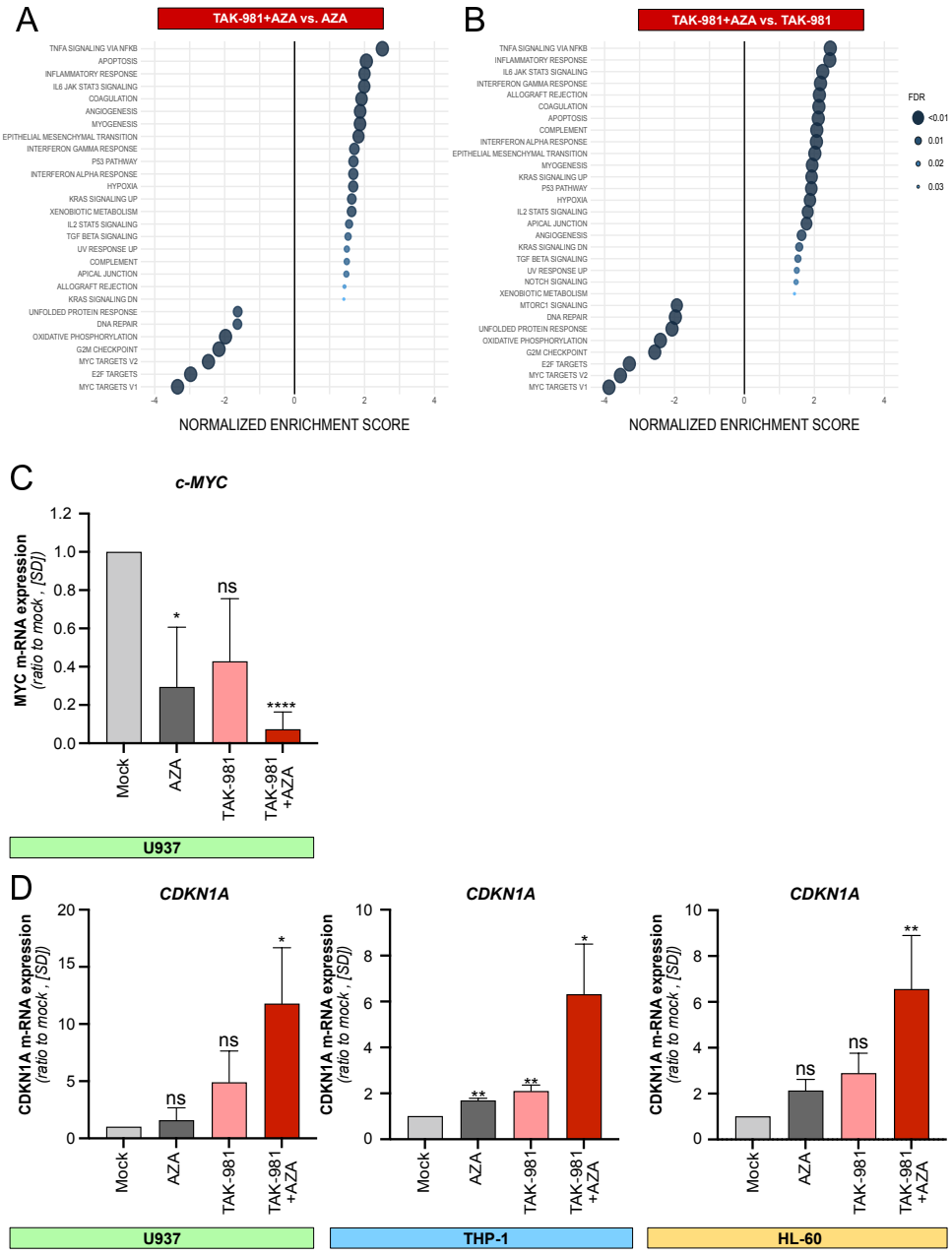
Supplementary Figure 2. Patient cells with abnormal karyotypes and from the M4/M5 FAB subtypes are more sensitive to TAK-981+AZA. Patient (n = 17) bone marrow mononuclear cells were treated for 3 consecutive days (Day 1, 2, 3) with TAK-981 (10 nM) and/or AZA (100 nM) and kept in culture. After 8 days, cells were collected and the number of CD45+ cells was analyzed by flow cytometry in each condition and compared to the mock-treated condition. For each group, plain lines represent the median value, and dotted lines are the quartiles. Groups were compared using unpaired t-test after sorting the patients depending on their karyotype (normal or abnormal)(A) or FAB subtype (M1/M2 or M4/M5).



Supplementary Figure 3: TAK-981 and AZA combination has a higher anti-leukemic activity than monotherapies *in vivo.* A) NSG mouse (8-9 mice/group) were treated with TAK-981 (15 mg/kg) for the indicated times. Extracts from bone marrow cells were used in microbeads-based assay to monitor the activity of SUMOylation enzymes. **B)** NSG mouse treatment schedule for experiments conducted in Figure 2A and 2B. **C)** Quantification, as photons/second/cm^2/sr of tumor burden evolution monitored by luminescence intensity in mice (5/group) injected with bioluminescent THP-1 cells. **D)** Schematic representation of mouse treatment schedule for experiments conducted in Figure 2C and 2D.

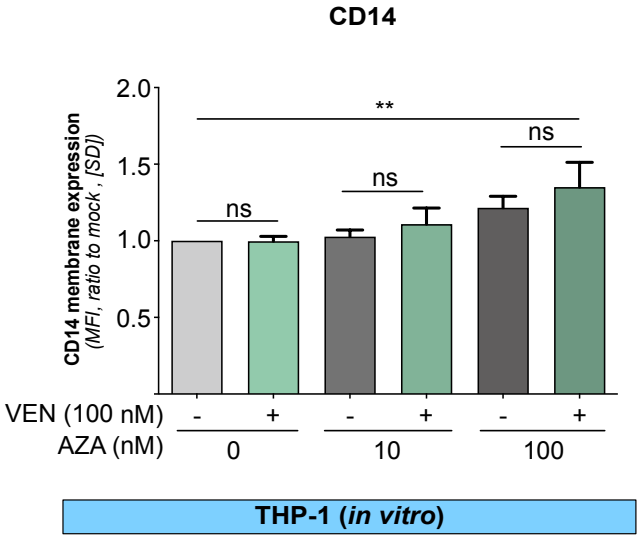


Supplementary Figure 4: TAK-981 and AZA combination has higher anti-leukemic activity than monotherapies on U937 cells *in vivo*. A) NSG mice treatment schedule for experiments conducted in B, C and D. **B-C)** Quantification as photons/second/cm2/sr (B) and relative luminescence units (C) of tumor burden evolution monitored by luminescence intensity in mice (6/group) injected with bioluminescent U937 cell line. **D)** Overall survival after treatment start of mice injected with bioluminescent U937 cell line was estimated in each group and compared with Kaplan-Meier method and log-rank test. **E)** NSG mice were injected with primary cells from one AML patient (PDX#3). After engraftment, mice were treated with AZA and/or TAK-981 and euthanized at day 9. The total number of human CD45+ cells (hCD45) was estimated by flow cytometry in spleen and compared to the mean number of cells collected in the mock-treated group of mice. For each group, plain lines represent the median value, and dotted lines are the quartiles. Groups were compared using Ordinary one-way ANOVA test.



Supplementary Figure 5: Gene expression signatures in U937 cells treated with TAK-981 and AZA

A, B) GSEA were performed using Hallmark datasets on the RNA-Seq data obtained from U937 cells. All pathways significantly enriched upon TAK-981+AZA compared to AZA (A) or TAK-981 (B) are shown (abs(NES)>1, p<0.05 and FDR<0.05). C) mRNA expression of *c-MYC* was analyzed by qRT-PCR in U937 cells treated for 72 hours with 10 nM AZA and 10 nM TAK-981. Results were normalized to *GAPDH* mRNA levels and expressed as ratio to mock-treated cells (n=5, mean +/- SD, one-way ANOVA). D) mRNA expression of *CDKN1a* was analyzed by qRT-PCR in U937 cells treated for 72 hours with 10 nM AZA and 10 nM TAK-981, THP-1 and HL-60 treated with 100 nM AZA and 10 nM TAK-981 (n=5 for U937, n=4 for THP1, n=3 for HL-60, mean +/- SD, one-way ANOVA)



Supplementary Figure 6: Effect of VEN+AZA on THP1 differentiation. Membrane expression of CD14 was measured by flow cytometry on THP-1 treated with AZA (10 or 100 nM) and 100 nM VEN or the drug combination for 72 hours. MFI were normalized to that of mock-treated cells (n=3, mean +/- SD, One-way ANOVA test)

Supplementary Tables (see excel files)

Supplementary Table 2: Clinical characteristics of the patient samples used in this study.

Supplementary Table 3: Raw data for the RNA-Seq analysis of U937 cells treated with TAK-981, AZA or TAK-981+AZA. The comparison between each experimental condition (3 biological replicates) is provided as Fold Changes and associated p-values.

Supplementary Table 4: Gene Set Enrichment Analysis of the U937 RNA-Seq data. GSEA analysis were performed on the Hallmarks and Gene Ontology Biological Process. For each gene signature, Enrichment Scores (ES) and Normalized Enrichment Scores (NES) as well as the associated p-values and False Discovery Rates (FDR) are provided for TAK-981-, AZA- or TAK-981+AZA versus mock-, AZA- and TAK-981-treated cells.