

Supplemental materials and methods

1. Cell lines and cell culture

All AML cell lines were tested to assess for the presence of mycoplasma and validated by short tandem repeat analysis. All cells were stored in liquid nitrogen. HEL and MOLM-16 were cultured in RPMI 1640 supplemented with 20% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 ug/mL streptomycin. The cells were grown at 37°C in a humidified atmosphere containing 5% carbon dioxide and were discarded after passage twenty. The AML cell lines HEL, MOLM-16, MEG-01, and CMK were purchased from Zhejiang Meisen Cell Technology Co.,Ltd. The THP-1 and M07E cell lines were purchased from Procell Life Science &Technology Co.,Ltd. The cell lines U937, HL60, Kasumi-1, NB4, MV4-11, and K562 were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co.,Ltd. The cell lines were tested for mycoplasma using the Myco-Lumi™ Luminescent Mycoplasma Detection Kit for High Sensitivity Instrument and were validated by Jianda Biotechnology Co.,Ltd

using a short tandem repeat analysis. 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Mononuclear blasts were isolated using a red blood cell lysis solution, and cell viability was assessed using the trypan blue exclusion assay. The cells were cultured in RPMI 1640 supplemented with 20% fetal bovine serum (NSERA), 100 ng/mL SCF, 100 ng/mL Flt-3, and 10 ng/ml IL-3 (Pepro Tech) at 37°C with a humidified atmosphere containing 5% CO₂.

2. Patients

From October 2013 to September 2022, the center received and treated a number of children with AML. For these children, we set the following clear inclusion and exclusion criteria to ensure the scientific and accurate study. The inclusion criteria mainly covered the following areas: first, the child must be no older than 15 years of age and have a first diagnosis of AML; second, the diagnosis must meet the World Health Organization criteria; and third, the child and his/her guardian must agree to and accept the hospital's treatment plan and sign an informed consent form before treatment. In addition, the child and his/her guardian must agree to participate in the

study, the study is subject to approval by the Ethics Committee of the Children's Hospital of Soochow University. The exclusion criteria included Fanconi anemia, other tumors, and therapy-related AML. At the same time, children who had received chemotherapy in other medical institutions, children who had received only short-term chemotherapy, radiotherapy or immunotherapy without a systemic chemotherapy regimen, and children who had abandoned treatment halfway through. We recruited 148 pediatric AML patients in this retrospective study from October 2013 to September 2022 in our center. We used PASS 15.0 software to adequate power to detect a pre-specified effect size. For two-sided hypothesis testing, the power of our sample size is 0.99. Data on the RNA sequencing results of all patients were available. The patients were treated with either low doses of chemotherapy or standard doses of chemotherapy for the induction therapy and consolidated by either high-dose-based cytarabine or allo-hematopoietic stem cell transplantation according to their risk classification.

This study was approved by the Ethics Committee of the Children's Hospital of Soochow University (No. 2017047-3) and was in accordance with Declaration of Helsinki.

Informed consent was obtained from the parents or legal guardians and participants.

3. In vivo Murine Modeling

All animal experiments were conducted in compliance with the animal care guidelines approved by the Institutional Laboratory Animal Care and Use Committee of Soochow University (SUDA20230802A05) and were in accordance with the ARRIVE guidelines. Five- to six-week-old NSG mice were purchased from Shanghai Model Organisms Center, Inc. 2×10^6 HEL cells overexpressing firefly luciferase were injected into the tail vein of NSG mice. We randomly assigned 28 NSG mice to four groups as experimental groups. The technicians who did immunohistochemical and blood routine tests did not know the specific grouping. The subjects were unaware of the grouping of the trials either. We used a single-blind method in which subjects were unaware of the grouping of the trials. Fourteen days after leukemia cell injection, the mice were treated with either vehicle control, IDA alone (0.5 mg/kg iv, on days 14-16), ANA alone (5 mg/kg oral gavage, on days₂ 14-28), IDA (0.5 mg/kg iv, on days 14-16)

and ANA (5 mg/kg oral gavage, on days 14-28). The sample size of each group is 7 NSG mice. The experimental mice were monitored closely for clinical signs of leukemia such as weight loss and hind limb paralysis. At the end of each experiment, bioluminescence images were taken, and the survival time was observed. When vehicle control mice displayed signs of terminal leukemic disease, they were euthanized by inhalation of CO₂. Then, their livers and spleens were weighed and collected for subsequent experimental procedures, including paraffin block, HE staining, and immunohistochemistry staining. Primary antibodies against human CD45 were used according to the manufacturer's instructions.

4. Inhibitors and Chemotherapeutics

For in vitro studies, anagrelide (ANA), 17- β -estradiol (E2), idarubicin (IDA), homoharringtonine (HHT), cytarabine (Ara-c), etoposide (VP-16), decitabine (DEC), cladribine (Clad) and Z-VAD-FMK were purchased by Selleck; 6-(4-(diethylamino)-3-nitrophenyl)-5-methyl-4,5-dihydropyridazin-3(2H)-one (DNMDP), zardaverine and daunorubicin (DNR) were purchased by MedChemExpress; and nauclefine was purchased by Shanghai Yuanye Biotechnology Co., Ltd. Powder was dissolved in DMSO in 10–50 mM stock solutions and stored at –20°C. For in vivo studies, ANA was dissolved in DMSO at 4 mg/mL, while IDA was dissolved in DMSO at 0.5 mg/mL; all dissolved drugs were aliquoted and stored at –20 °C. Then, the substance was freshly formulated on the day of dosing as 5 mg/kg ANA and 0.5 mg/kg IDA.

5. Cell viability assay and colony assays

The compounds were diluted in DMSO (<0.05%) and dispensed into 96-well plates. The cells (2x10⁴ cells/well) were plated and treated for 72 hours in triplicates for each experimental condition. Each well was incubated with 20 μ l of CCK-8 (Sigma), and the plate was incubated for 2-4 hours at 37°C in a humidified, 5% CO₂ atmosphere. Absorbance was measured on a Synergy H4 Hybrid Microplate Reader (Bio-TEK Instruments) and corrected by subtracting the absorbance of blank wells from that of the wells containing the CCK-8 reagent and

media only. The IC₅₀ values were determined by creating dose-response curves (GraphPad Prism 8.0.2). CCK8 readings at specified time points were normalized to vehicle control readings, and the IC₅₀ concentrations were calculated. The cells of the experimental and control groups were inoculated in a soft agar medium (Sigma), cultured for 9-14 days, and stained with Giemsa, and the number of colonies was counted. Serial colony-forming assay: primary cells from AML#6 were treated with PBS (control), 5uM ANA, 0.005uM IDA and 5uM ANA combined with 0.005uM IDA for 72h. Then, 5,000 cells from each group were seeded into the methycellulose medium (Methocult, M3434; STEMCELL Technologies) with 100ng/ml human SCF, 100ng/ml human Flt-3 and 10ng/ml human IL-3. Colonies were scored after 10-12 days of incubation at 37°C in a 5% CO₂ atmosphere. For serial replating, cells were collected, washed with PBS and replated (5,000 cells per plate) into new methycellulose medium for every other 10-12 days.

6. Cell-cycle analysis

The cells were treated with the medicine for 72 hours, fixed, permeabilized overnight by adding 70% ethanol, and precooled after collection. The samples were washed and resuspended in cold PBS with 25 µg/ml RNase A and 1.5 µmol/l propidium iodide (PI) and incubated in the dark for 30 min at 37 °C. The samples were examined in a Beckman Gallios™ flow cytometer (Immunotech Beckman Coulter) and analyzed by Flow Jo V10.

7. Cell apoptosis analysis

The cells were treated with medicine at the predetermined concentrations in six-well plates at 37°C. After 72 hours of incubation, the cells were collected and stained with Annexin V-FITC and PI following the instructions of the FITC-Annexin V Apoptosis Detection Kit (BD Biosciences). Apoptosis was analyzed using Flow Jo V10 software.

8. Enzyme-linked immunosorbent assay analysis

After the indicated drug treatment, the levels of IL-1β and IL-18 were measured using the Human IL-1 β ELISA Kit and Human IL-18 ELISA Kit (NEOBIOSCIENCE) according to the recommended protocol. Each sample was tested in triplicates.

9. RNA extraction and quantitative real-time PCR

Total RNA was isolated from the cells using the TRIzol extraction reagent (Invitrogen). cDNA was synthesized using PrimeScript RT Master Mix (TaKaRa Bio Inc.) for quantitative real-time PCR. Quantitative real-time PCR was performed using SYBR Green PCR kit (Takara Bio Inc.) and analyzed using an ABI 7500 Sequence Detection System. The expression of each gene was normalized to that of GAPDH. All primer sequences were designed using the Primer website (<https://pga.mgh.harvard.edu/primerbank/>):

PDE3A:5'CCACGGCCTCATTACCGAC-3'(forward)

5'TTGCTCACGGCTCTCAAGG-3'(reverse).

GSDME:5'TGCCTACGGTGTTCATTGAGTT-3'(forward)

5'TCTGGCATGTCTATGAATGCAAA-3'(reverse).

GAPDH:5'ATCATCCCTGCCTCTACTGG-3'(forward)

5'CCCTCCGACGCCTGCTTCAC-3'(reserve).

10 . Western blot analysis

Western blotting analyses were performed using standard techniques. Proteins were detected using primary antibodies against PDE3A (Abcam, ab244337), C-myc (Cell Signaling Technology, 9402), cyclin D1 (Cell Signaling Technology, 2978), CDK2 (Cell Signaling Technology, 18048), CDK4 (Cell Signaling Technology, 12790), CDK6 (Cell Signaling Technology, 13331), P53(Cell Signaling Technology,9282), P21(Cell Signaling Technology,2947), P27(Cell Signaling Technology,3686), GAPDH (Bioworld Technology Inc., AP0063) , P-SRC (Cell Signaling Technology, 12432), SRC (Cell Signaling Technology, 2109), P-ERK1/2 (Cell Signaling Technology, 4370), ERK1/2 (Cell Signaling Technology, 9194), P-P38 (Cell Signaling Technology, 4511), P38 (Cell Signaling Technology, 8690), P-JNK (Cell Signaling Technology, 4668), JNK (Cell Signaling Technology, 9252), ERK5 (Cell Signaling Technology, 3372), P-ERK5 (Cell Signaling Technology, 3371),

PAK1 (Proteintech Group, Inc., 21401-1-AP), DUSP16(Cell Signaling Technology,5523), EGR1 (Santa Cruz Biotechnology, C1513), HMGCS1 (Proteintech Group, Inc., 176434-1-AP), NLRP3 (Abcam,

ab263899), caspase-3 (Cell Signaling Technology, 9664), caspase-8 (Cell Signaling Technology, 9746), GSDME (Abcam, ab215191), caspase1 (Cell Signaling Technology, 3866), caspase4 (Cell Signaling Technology, 42264), caspase5 (Cell Signaling Technology, 46680), and caspase11 (Novus Biologicals, NB120-10454).

11 . Calculation of Combination Index

The combination index (CI) was calculated using the CalcuSyn software according to the Chou–Talalay method. CI was used for the quantification of synergism or antagonism of two drugs, where $CI < 1$, $= 1$, and > 1 indicated synergism, additive effect, and antagonism, respectively.

12. RNA-seq analysis and data processing

RNA-Seq was performed according to the protocols described by Novogene. Total RNA was isolated using a TRIzol reagent. RNA purification, library construction, and sequencing were performed using the Novogene software. The gene expression profiles in HEL cells treated with 1 μ M ANA, 0.005 μ M IDA, 1 μ M ANA+0.005 μ M IDA or the control for 72 hours were identified by RNA-seq. The gene expression profiles in primary cells from AML#6 treated with 5 μ M ANA, 0.005 μ M IDA, 5 μ M ANA+0.005 μ M IDA or the control for 72 hours were identified by RNA-seq. Next, we calculated the gene expression levels and detected the presence of differentially expressed genes using DESeq2 (adjusted $P < 0.05$, absolute value $\log_2$ fold-change > 0.5). For enrichment analysis, differentially expressed genes were analyzed using the GSEA software (cluster Profiler package 4.0.5 in R 4.1.1). The genes with an adjusted P-value of < 0.05 were selected for Kyoto Encyclopedia of Genes and Genomes analysis and heatmap construction.

Supplementary Figure1

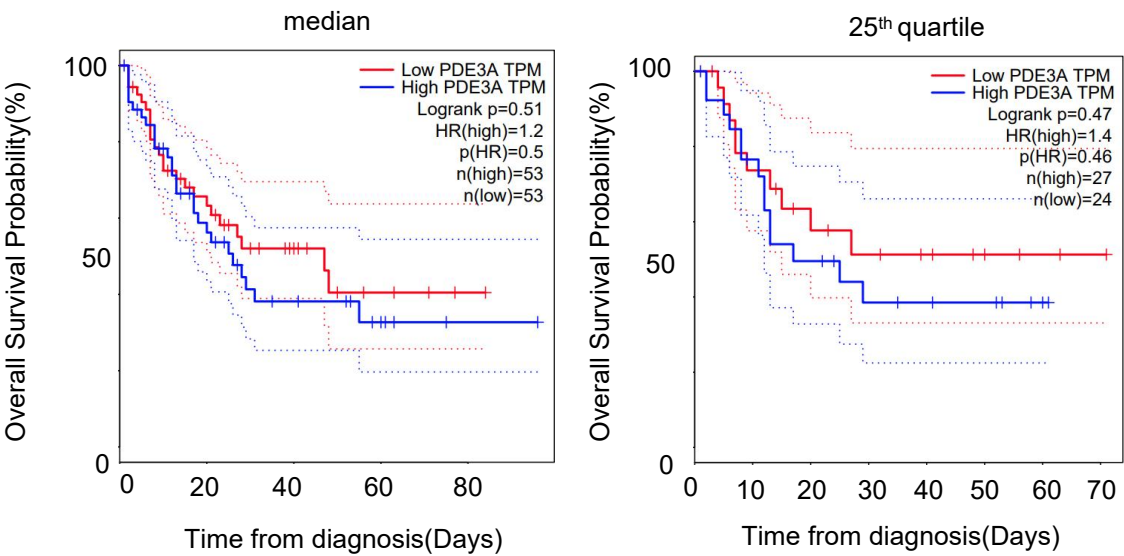


Figure S1 AML patients with high expression of PDE3A had shorter OS than those with low expression of PDE3A OS of AML patients in the LAML data set stratified according to PDE3A expression status. Survival curves were analyzed using the Kaplan-Meier method, and P-values were determined using the log-rank (Mantel-Cox) test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS, not significant

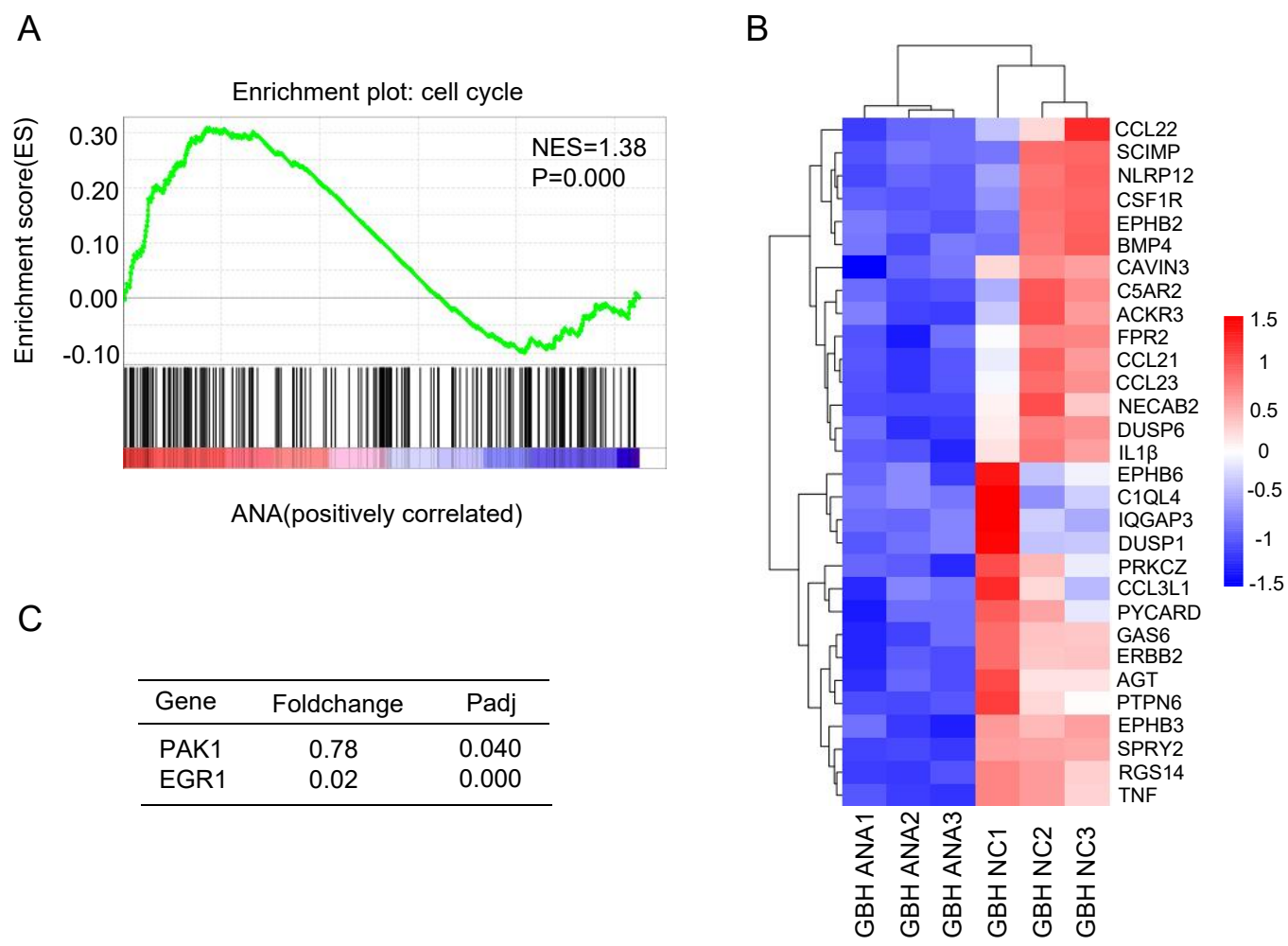


Figure S2 ANA treatment on bone marrow mononuclear cells from newly diagnosed AML patient 6# induced changes in cell cycle-related proteins and down-regulation of drug resistance-related proteins (A) Significantly enriched GSEA signatures in the transcriptional profile of primary cells from AML patient#6 following the treatment with ANA. The normalized enrichment score (NES)and P-value are shown. **(B)**Heat map of differentially expressed genes in primary cells from AML patient#6 in the “ERK1/2 cascade” following the treatment with ANA. Each column represents a sample each row represents a gene, blue represents the downregulation of genes, and red represents the upregulation of genes.**(C)** The adjusted P-value and fold change of the related differentially expressed genes are shown in the table. Data are presented as the mean $\pm$ SD (n=3).

Supplementary Figure3

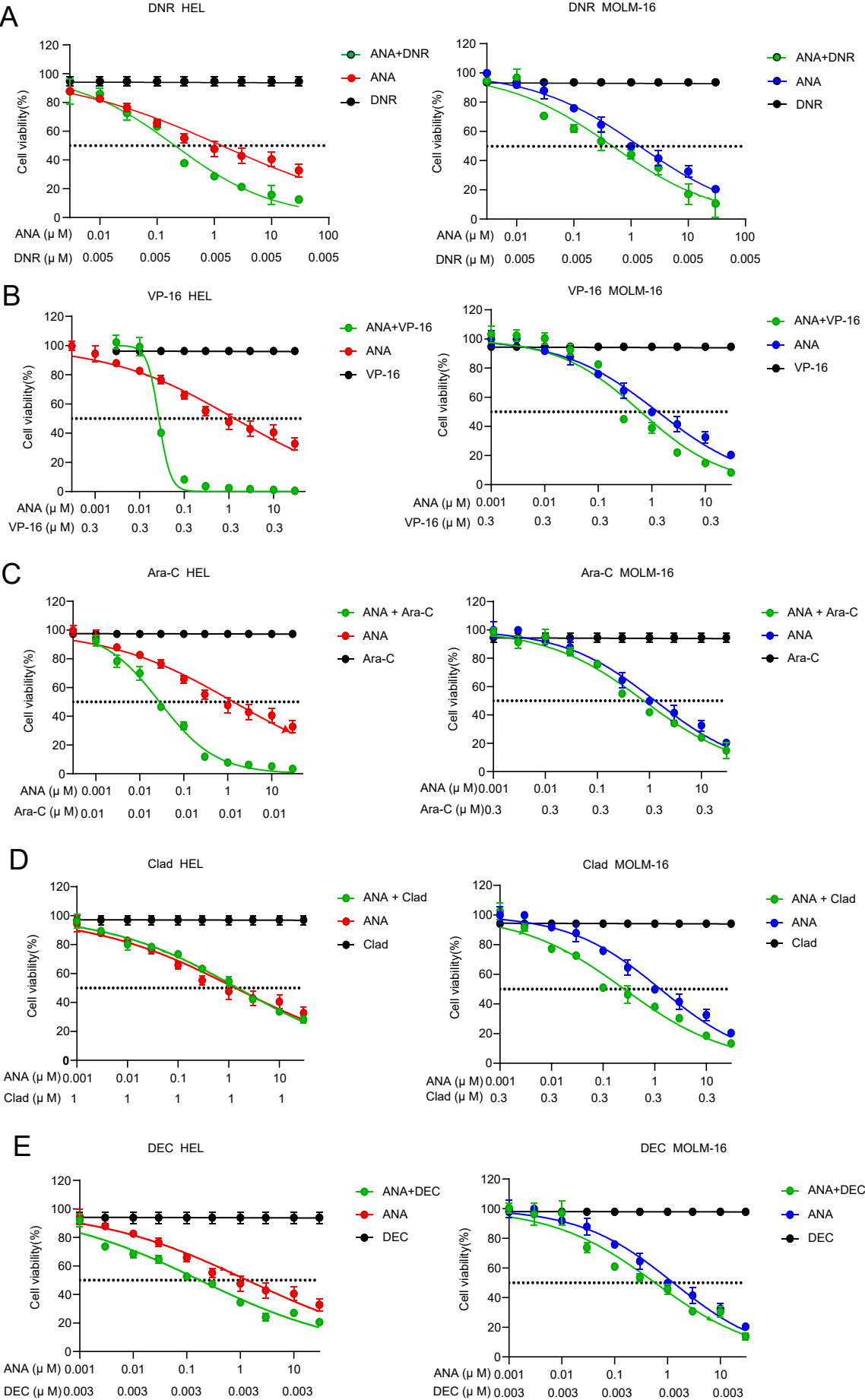
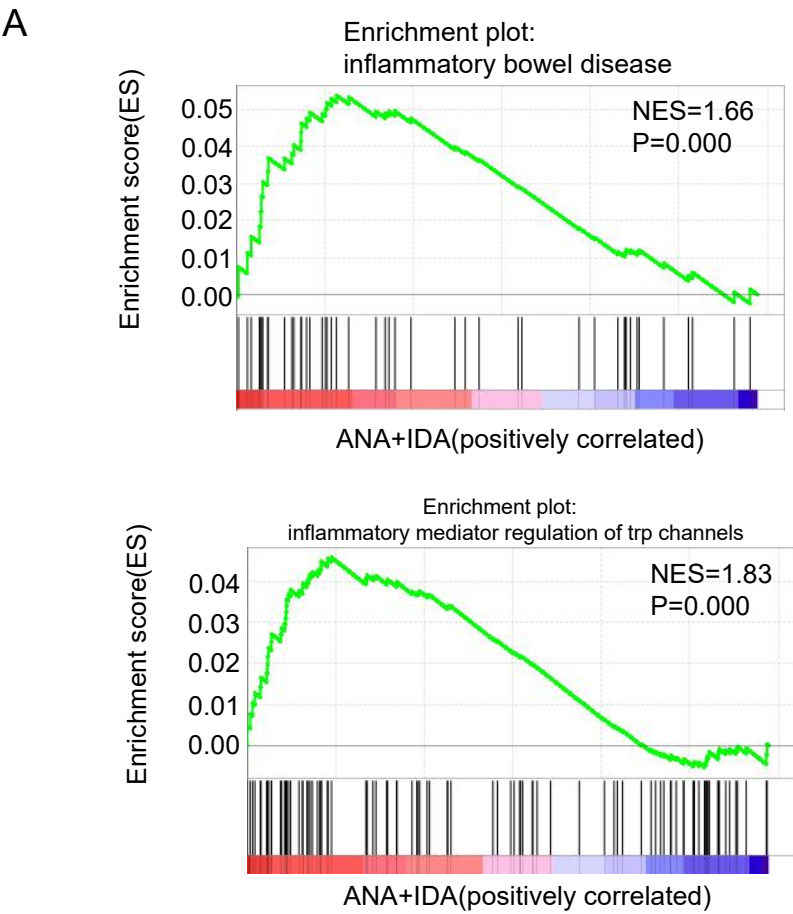


Figure S3 The synergistic effect of ANA combined with common chemotherapeutic drugs (A-E) HEL and MOLM-16 were treated with the indicated concentrations of ANA and DNR/VP-16/Ara-C/clad/DEC for 72 hours, and then subjected to CCK-8 assay for detection of the anti-proliferative effect. Data are presented as the mean $\pm$ SD (n=3). Comparisons were evaluated using the two-tailed Student's t test, and multiple groups were analyzed using a one-way analysis of variance. *P< 0.05, **P < 0.01, ***P < 0.001, ****P <0.0001. NS, not significant

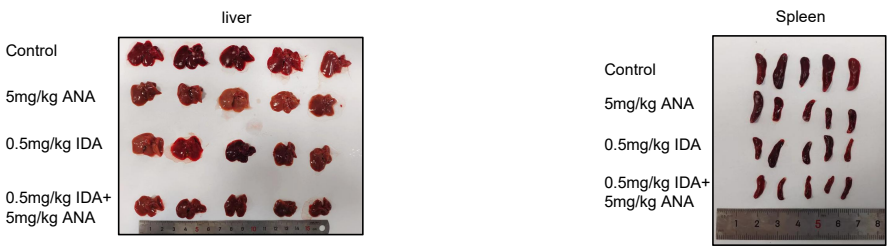


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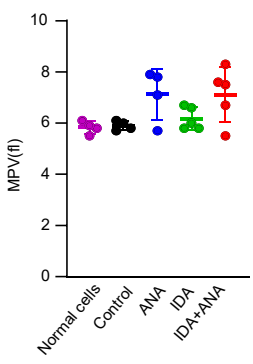
Gene	Foldchange	Padj
GSDME	2.59	0.000
IL1β	13.45	0.000
Caspase3	1.54	0.000

Figure S4 (A) ANA-IDA treatment on bone marrow mononuclear cells from newly diagnosed AML patient 6# induced positive enrichment of the inflammatory related pathway and up-regulation of pyroptosis-related proteins (A) Significantly enriched GSEA signatures in the transcriptional profile of primary cells from AML patient#6 following the combination treatment with IDA and ANA. The normalized enrichment score (NES) and P-value are shown.(**B**) The adjusted P-value and fold change of the related differentially expressed genes are shown in the table. Data are presented as the mean ± SD (n=3).

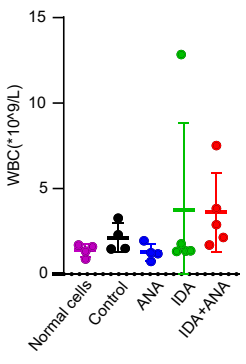
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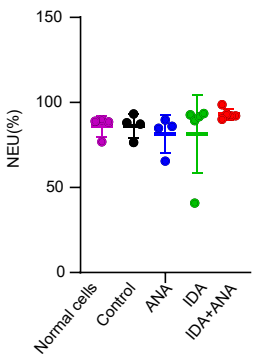
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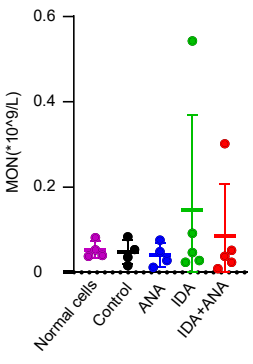
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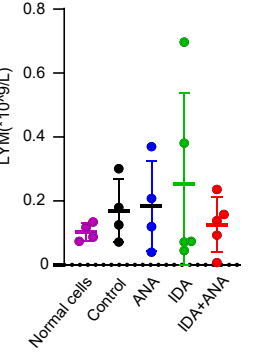
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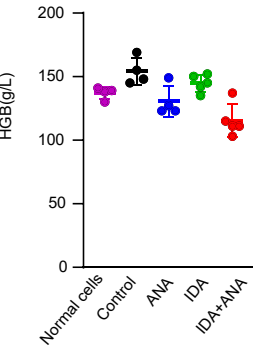
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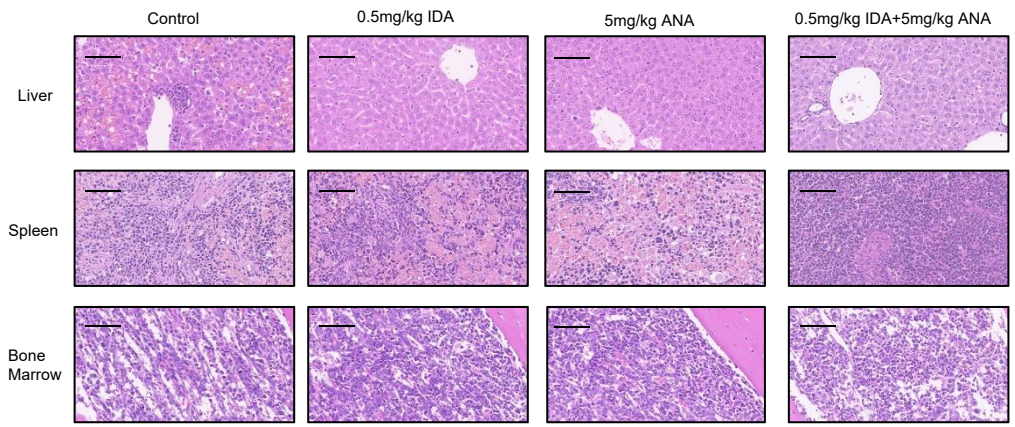


Figure S5 ANA-IDA treatment exerting synergistic antileukemia effects on AML xenograft mouse models **(A)** Livers and spleens of four groups of mice. **(B-G)** Blood routine of experimental mice in each group. **(H)** HE staining of histologic sections of the bone marrow, liver, and spleen in experimental mice. Data are presented as the mean $\pm$ SD (n=3). Comparisons were evaluated using the two-tailed Student's t test, and multiple groups were analyzed using a one-way analysis of variance. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS, not significant