

A Novel Strategy to Target Metabolic Dependencies in Acute Myeloid Leukemia

Nithya Balasundaram^{1,2}, Hamenth Kumar Palani¹, Arvind Venkatraman¹, Yolanda Augustin³, Shruthi Pichandi¹, Clement Regnault⁴, Majeela Solomon¹, Abirami Rajasekaran¹, Mohammed Yasar¹, Swathy Palani Kumar¹, Reeshma Nair Radhakrishnan¹, Anu Korula¹, Uday Prakash Kulkarni¹, Eunice Sindhuvi Edison¹, Poonkuzhali Balasubramanian¹, Biju George¹, Aby Abraham¹, Sanjeev Krishna^{3, 5-7, *}, Vikram Mathews^{1*}.

¹Department of Haematology, Christian Medical College, Vellore, India.

²Laboratory of Cellular Metabolism and Microenvironment, De Duve Institute, UC Louvain, Brussels, Belgium.

³Clinical Academic Group in Institute for Infection & Immunity, St George's University of London, London, United Kingdom

⁴MVLS Shared Research Facilities, University of Glasgow, Glasgow, United Kingdom.

⁵St George's University Hospitals NHS Foundation Trust, United Kingdom

⁶Institut für Tropenmedizin, Universitätsklinikum Tübingen, Tübingen, Germany

⁷Centre de Recherches Médicales en Lambaréné (CERMEL), Lambaréné, Gabon

* These authors equally contributed to the work.

Supplemental Methods:

Colony Formation Unit (CFU) assay: CD34⁺ hematopoietic stem and progenitor cells (HSPCs) were then enriched from healthy donors' mobilized peripheral blood cells through positive selection using the CD34 MicroBead Kit (Miltenyi Biotec) according to the manufacturer's protocol. Following purification, cells were resuspended in hematopoietic stem cell colony-forming unit (HSC-CFU) medium. 10⁴ CD34 positive cells, single-cell colony formation, the cell suspension was diluted and seeded into 96-well plates so each well contained a single CD34⁺ cell. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO₂ for a period of 14 days to allow colony formation and differentiation. After 14 days, the cells were analysed for colonies and to identify the type of colonies, the cells were stained with a lineage-specific antibody cocktail comprising CD235a (erythroid lineage), CD14 (monocytic lineage), and CD15 (granulocytic lineage). Flow cytometry analysis was performed to enumerate colonies and evaluate lineage commitment based on surface marker expression per the manufacturer's protocol (StemMACS™ HSC-CFU Assay Kit, Human).

Semi-quantitative real-time polymerase chain reaction: Total RNA was extracted using Trizol reagent (Invitrogen Carlsbad, CA, USA). 500ng of the extracted RNA was converted into cDNA

5 times, and the protein bands were detected by the standard chemiluminescence method (ThermoFisher Scientific).

Wright-Geimsa Staining: 50K U937 cells in one hundred microliters were used to make cytospin slides (500RPM for 5 minutes). The slides were then air-dried and fixed with one hundred per cent methanol for 10 minutes, washed and stained with Wright-Giemsa for 5 minutes, and washed with PBS, and the images were captured in an inverted light microscope using an oil immersion lens (Olympus).

Endogenous ROS measurement: 5×10^5 treated/control cells were stained with 5uM of DCFDA (Sigma) for 15 minutes at 37 degrees. The stained cells were washed twice with phosphate-buffered saline (PBS) at 500g for 5 minutes. The cell pellet was resuspended in PBS, and the fluorescence was measured using Flow cytometry (Beckman Coulter Navios).

Mitochondrial Calcium Measurement: 5×10^5 treated/control cells were stained with 5uM of Rhod-2 AM (ThermoFisher Scientific) for 20 minutes at 37 degrees. The stained cells were washed twice with phosphate-buffered saline at 500g for 5 minutes. The cell pellet was resuspended in PBS, and the fluorescence was measured using Flow cytometry (Beckman Coulter Navios).

Untargeted Metabolomics: 5 million cells were cultured and treated with DMSO, ATO, ART and the combination of ATO+ART. After 24 hours of treatment the cells were collected and quenched in a dry ice/ethanol bath followed by centrifugation for 10 minutes at 1000g, 4 degrees. The supernatant was removed completely, and the pellet was re-suspended in 200ul of (1:3:1) ice-cold Chloroform: Methanol: Water mixture to extract the metabolites and incubated for 1 hour in a rocker at 4 degrees. Then, the sample was spun at 13000g for 3 minutes at 4 degrees and the supernatant was collected and stored at -80 degrees. Liquid chromatography-mass spectrometry (LC/MS) for the extracted metabolites was performed using a pHILIC column in a Thermo Orbitrap at the Glasgow polyomics facility, University of Glasgow, UK.

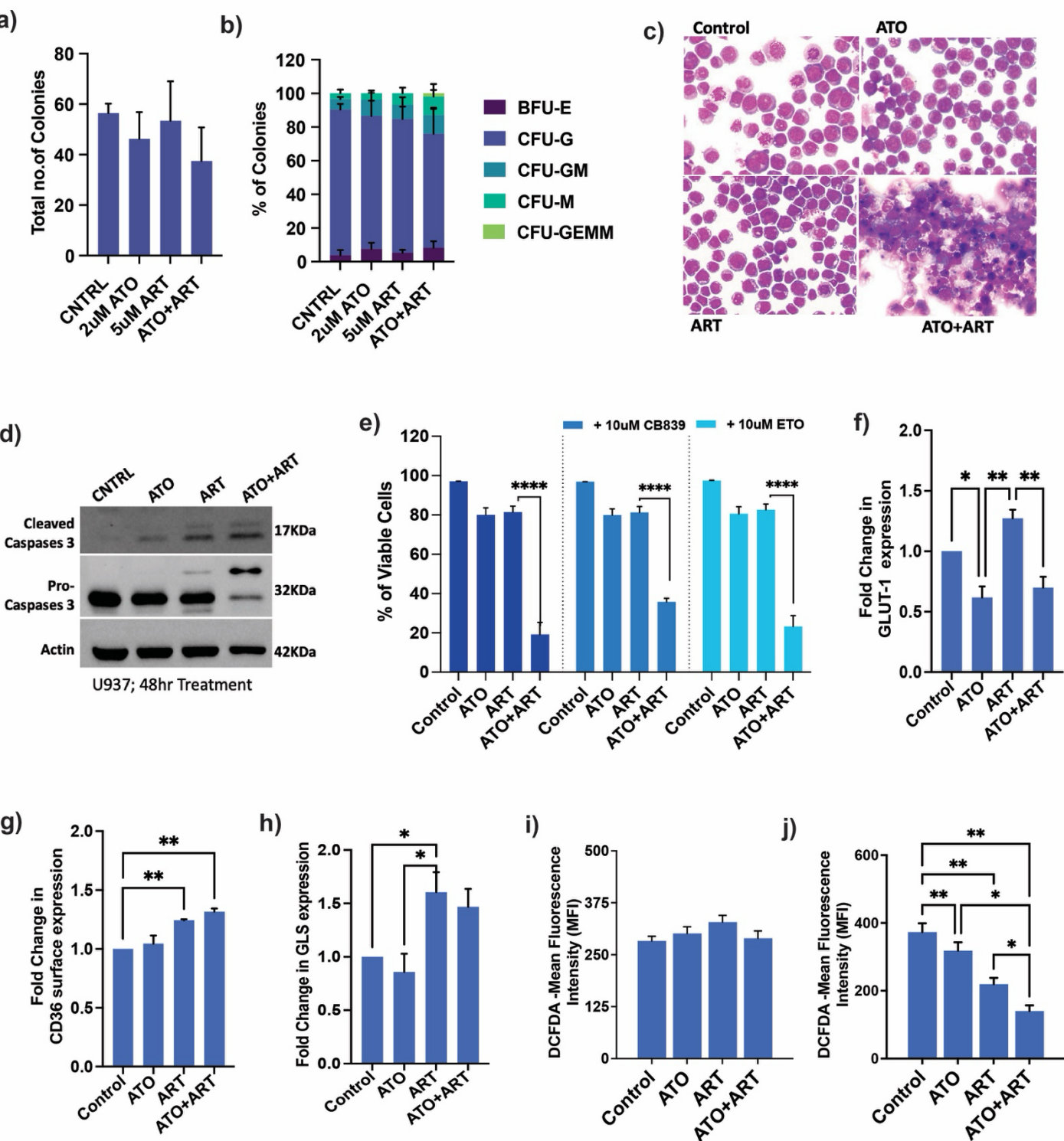
Supplementary Figure 1:

- a) The total number of colonies by the normal CD34-positive cells treated with ATO, ART, and ATO+ART at the end of 14 days cultured in the Methocult (H4434) (n=5; ATO =2uM; ART = 5uM)
- b) Types of colonies formed by the normal CD34 positive post 14 days of ATO, ART and ATO+ART were characterized using FACS (StemMACS HSC-CFU Assay Cocktail). (n=5; ATO = 2 μ M; ART = 5 μ M).
- c) Immunoblot of full-length caspase-3 (32kDa) and cleaved Caspase-3 (17kDa) in U937 cells treated with ATO and ART for 48 hr (n=3; ATO = 2 μ M; ART = 5 μ M).
- d) Representative images of Wright-Giemsa-stained U937 cells treated with ATO, ART, and ATO+ART for 24h (ATO=2uM; ART=5uM).
- e) Viability of U937 cells treated with ATO, ART and ATO+ART in combination with CB839 and etomoxir for 48h (n=6; ATO=2uM; ART=5uM and CB839= 10uM; ETO= 10uM).
- (f-h) Relative expression levels of GLUT-1, CD36 and GLS in U937 cells treated with ATO, ART and ATO+ART. (GLUT-1 and CD36 - n=3; GLS n=6; ATO=2uM; ART=5uM).
- (i-j) Reactive species levels of U937 cells treated with ATO, ART and ATO+ART for 6h and 24h (n=3; ATO=2uM; ART=5uM).

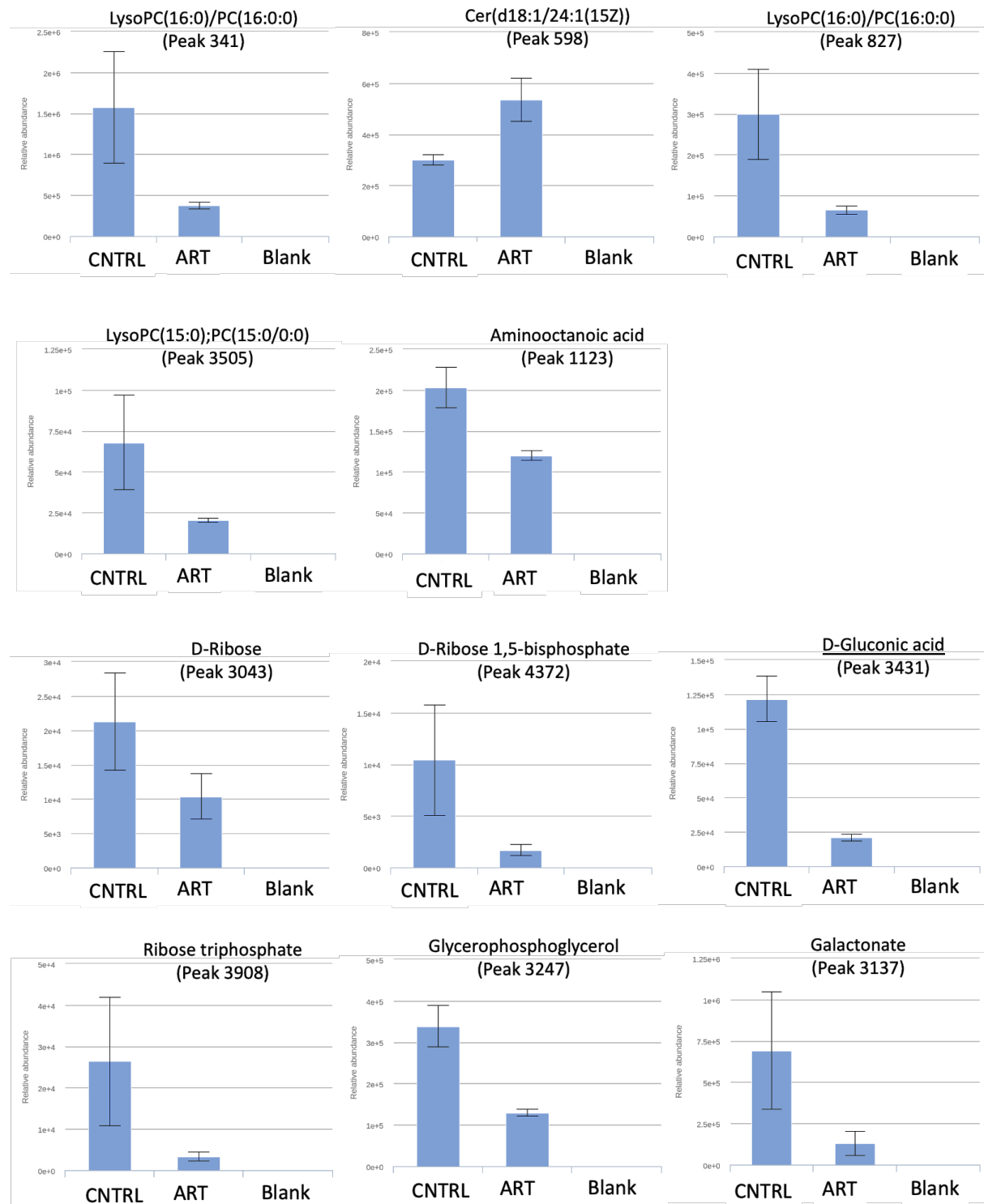
BFU-E: burst-forming unit-erythroid, CFU-G: colony-forming unit-granulocyte, CFU-M: CFU-macrophage, CFU-GM: CFU-granulocyte macrophage, CFU-GEMM: CFU-granulocyte erythrocyte macrophage megakaryocyte.

Data are presented as mean \pm SEM. n.s., $P > .05$; * $P < .05$; *** $P < .001$; **** $P < .0001$, with a two-tailed unpaired t-test or one-way analysis of variance.

Supplementary figure 1

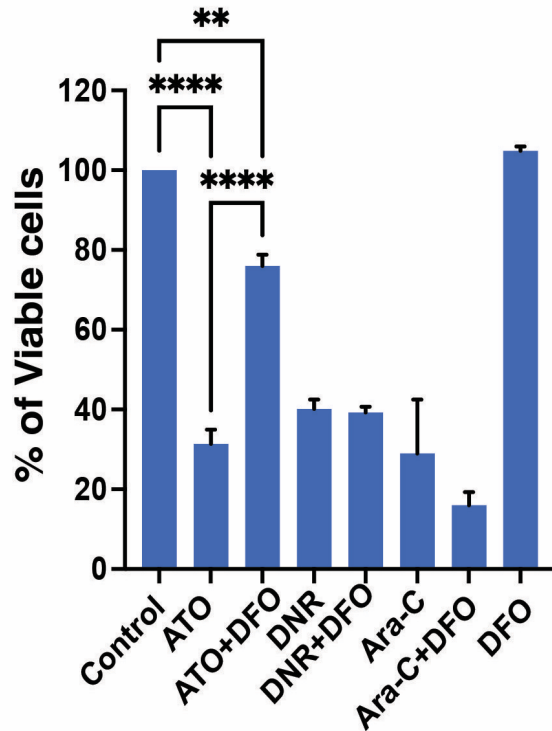


Supplementary figure 2:



Untargeted metabolomics of U937 cells treated with ART for 24h: Relative abundance of metabolites relating to lysophospholipids, and sugars. (n=3; ART=5uM; 24h)

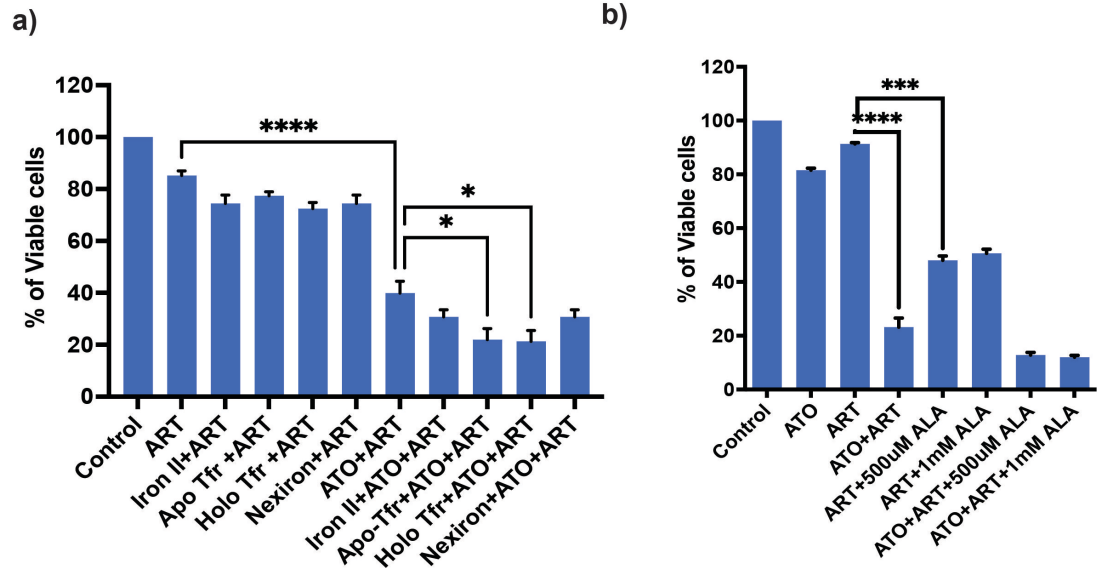
Supplementary figure 3:



Viability of ATO sensitive NB4 cells treated with ATO, DNR, Ara-C in combination with DFO for 48h (n=3; ATO=2uM; DNR = 40ng; Ara-C =400ng; DFO=20uM).

Data are presented as mean \pm SEM. n.s., $P > .05$; * $P < .05$; *** $P < .001$; **** $P < .0001$, with a two-tailed unpaired t-test or one-way analysis of variance.

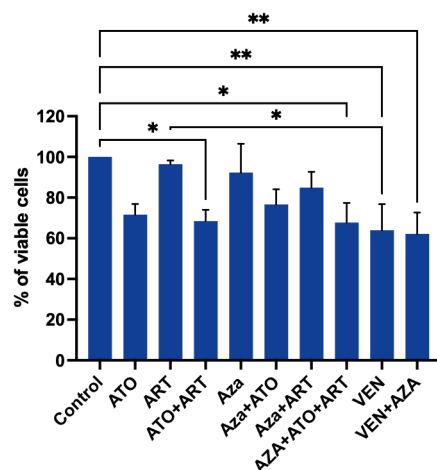
Supplementary Figure 4:



- a) Viability of U937 cells treated with different forms of iron in combination with ART and ATO+ART for 48h. (n=3; Iron II = 10ug; Apo and Holo Tfr = 50ug; Nexiron = 40ug).
- b) Viability of U937 cells treated with ALA in combination with ATO, ART, and ATO+ART for 48h. (n=4; ATO = 2uM; ART = 5uM and ALA = 500uM and 1mM)

Data are presented as mean \pm SEM. n.s., $P > .05$; * $P < .05$; *** $P < .001$; **** $P < .0001$, with a two-tailed unpaired t-test or one-way analysis of variance.

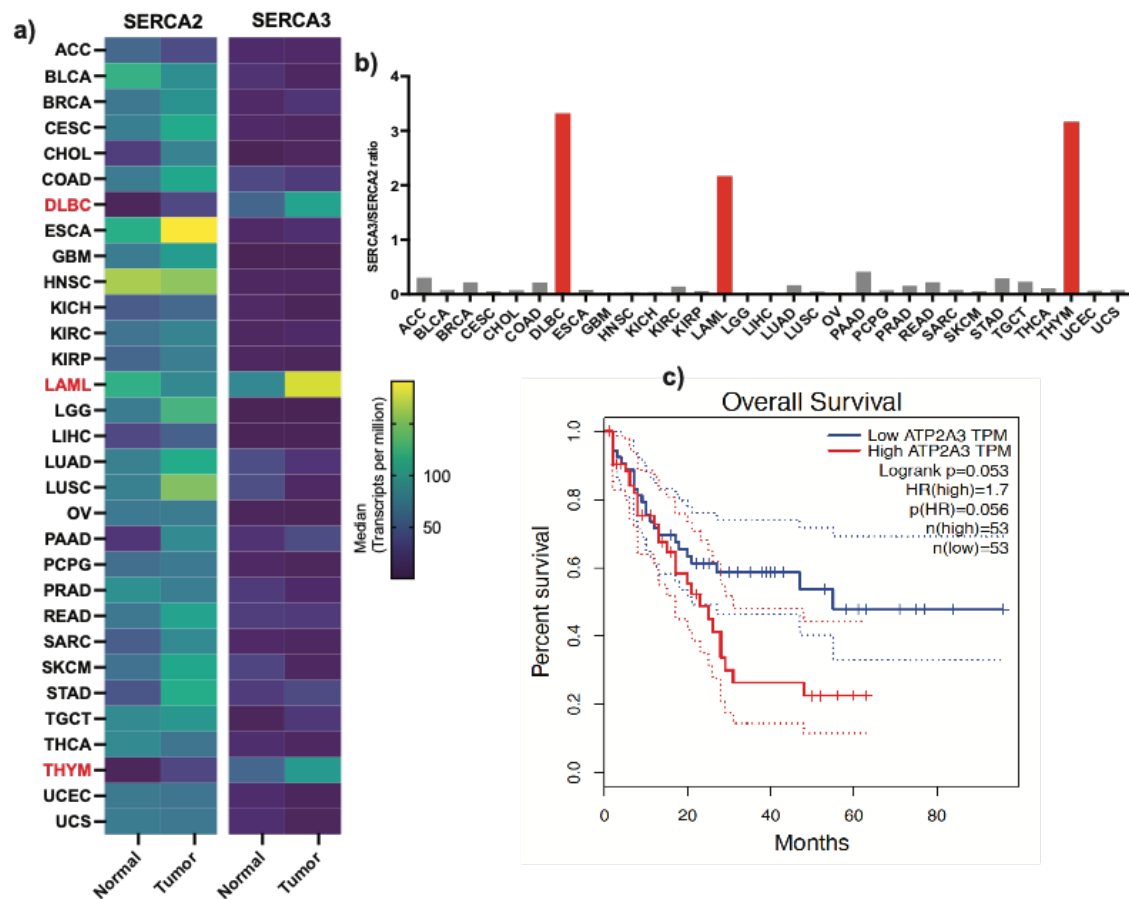
Supplementary Figure 5:



Viability of healthy peripheral blood mononuclear cells treated with ATO, ART, VEN and AZA for 48h. (n=5; ATO=2uM; ART =5uM; Aza =2.5ug; VEN = 500nM).

Data are presented as mean \pm SEM. n.s., $P > .05$; * $P < .05$; *** $P < .001$; **** $P < .0001$, with a two-tailed unpaired t-test or one-way analysis of variance.

Supplementary figure 6:



- SERCA2 and SERCA3 expression levels of normal and cancer tissues from the Cancer Genome Atlas TCGA dataset.
- SERCA3 to SERCA2 ratio for TCGA tumor types. Red bars highlight the hematological malignancies with a high SERCA3 to SERCA2 ratio. DLBC (Diffuse large B cell lymphoma), LAML (Acute myeloid leukemia), and THYM (Thymoma).
- Kaplan Meier survival curves of TCGA AML cohort stratified based on SERCA3 expression.

Supplemental Tables:

Table 1: Relative protein abundances enriched in the biotinylated ART pull-down of U937 cells (related to figure 2b and c).

Table 2: Metabolites peak intensities of ART-treated U937 and control cells (related to figure 2d and Supplementary figure 2).