

Metabolic adaptation drives arsenic trioxide resistance in acute promyelocytic leukemia

Nithya Balasundaram¹, Saravanan Ganesan¹, Ezhilarasi Chendamarai¹, Hamenth Kumar Palani¹, Arvind Venkatraman¹, Ansu Abu Alex¹, Sachin David¹, Swathy Palani Kumar¹, Nair Reeshma Radhakrishnan¹, Mohammed Yasar¹, Sanjeev Krishna², Anu Korula¹, Uday Kulkarni¹, Nancy Beryl Janet¹, Poonkuzhali Balasubramanian¹, Vikram Mathews¹.

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

²Institute for Infection and Immunity, St George's University of London, London, United Kingdom.

Supplemental Methods:

Measurement of intracellular ATO using atomic absorption spectrometer:

The intracellular levels of ATO was measured using well established and published protocols²⁹. Briefly, 2×10^7 cells were washed and suspended in RPMI media with 0.5 μ M concentration of ATO and incubated for 24 hours. The cell pellets were then washed twice in Ca²⁺ and Mg²⁺ free PBS (Stem Cell Technologies, Vancouver, Canada). The cell pellets were digested with a standard volume of suprapur nitric acid and hydrogen peroxide (Merck Millipore, MA, USA) (2:1 v/v) at 45°C for 16 hours. An aliquot of this final solution was analyzed using Atomic Absorption Spectrometer with EDL support equipment (Perkin Elmer, MA, USA).

Immunofluorescence for PML-RARA protein:

Cytospin slides of cell suspensions were made and fixed in 4% paraformaldehyde, followed by blocking using 5% goat serum and incubated with primary antibody (PML PG-M3 -Santa Cruz Biotechnologies, CA, USA) overnight at 4°C. The slides were washed and incubated with a secondary antibody conjugated with Alexa fluor 594 (Invitrogen, Carlsbad, CA, USA) for 1 hour, washed, air-dried, and counterstained with DAPI (4',6-diamidino-2-phenylindole) containing mountant (Vectashield, Burlingame, CA, USA). The images were acquired in a fluorescence microscope (Axioimager M1, Carl Zeiss, Germany) at $\times 100$ with oil immersion and images were analyzed using ISIS metasytem, (Metasystems GmbH, Altussheim, Germany).

Detection of PML-RARA by Immunoblotting:

Cell lines were harvested and lysed in RIPA buffer (Sigma) with complete protease inhibitors (Roche, Basel, Switzerland). The lysates were separated through SDS-PAGE and transferred to nitrocellulose membranes (Merck Millipore), blocked with 5% milk in TBS-T. After blocking the membrane was incubated with primary antibody RARA C-20 (Santa Cruz Biotechnologies, CA, USA) at 4°C overnight. Anti-rabbit secondary antibody conjugated with horseradish-peroxidase (Cell signaling technologies, MA, USA) was used to detect the fusion protein. The protein bands were detected by standard chemiluminescence method (Thermo Pierce Femto, Rockford, IL, USA). The images were captured using FluorChemQ system (Alpha Innotech, FL, USA) provided with Alpha View Software.

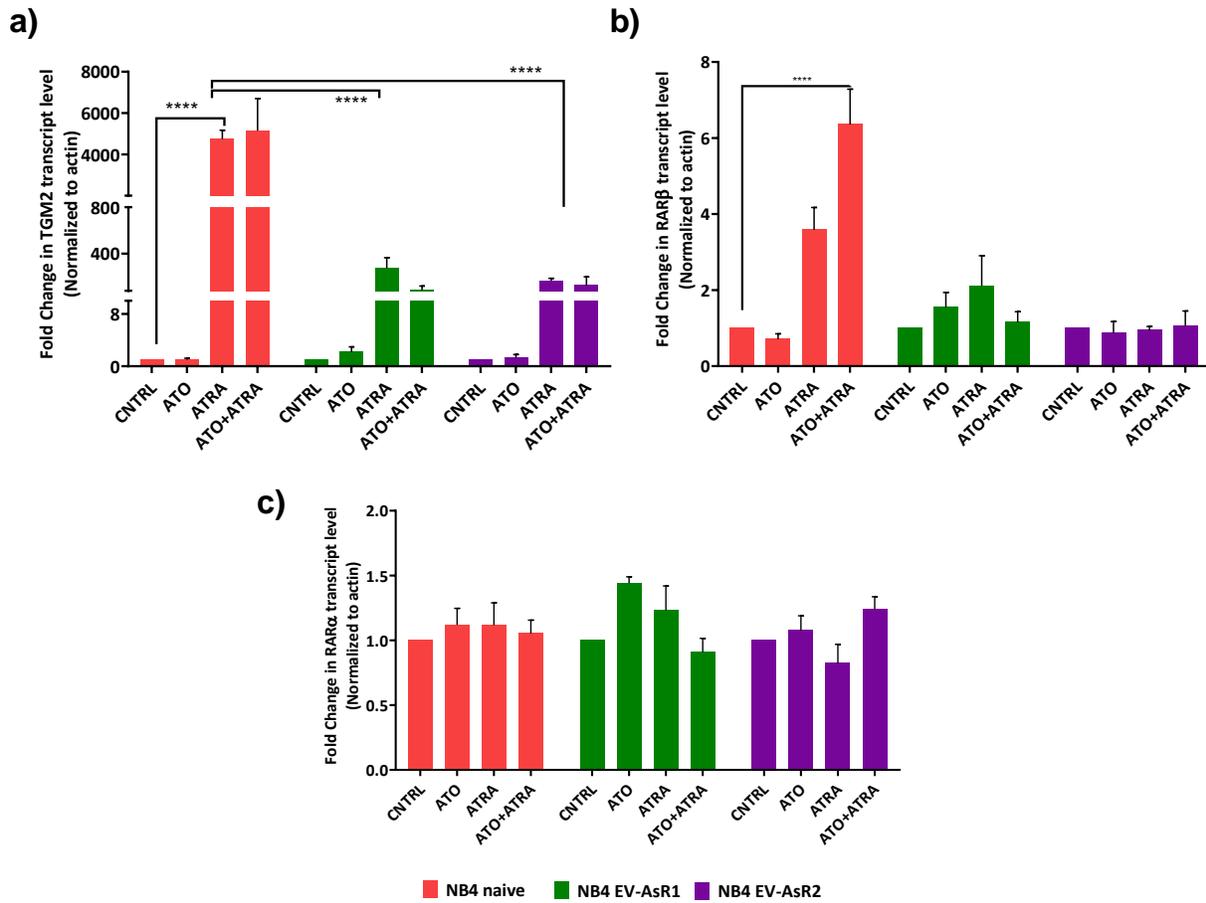
Endogenous Reactive oxygen species and mitochondrial membrane potential assay:

CellROX Green reagent and JC-1 (Invitrogen, Carlsbad, CA, USA) membrane potential dye was diluted in phosphate buffered buffer to obtain a final concentration of 5 μ M as working solution. Cells were incubated with Cell ROX for ROS and JC-1 for MMP for 20 minutes at 37°C. For ROS fluorescence was measured using flow cytometry (Beckman Coulter Navios, Brea, CA, USA) and MMP using Spectramax M4 (Molecular Devices, Sunnyvale, CA, USA).

mRNA and Real-time quantitative polymerase chain reaction:

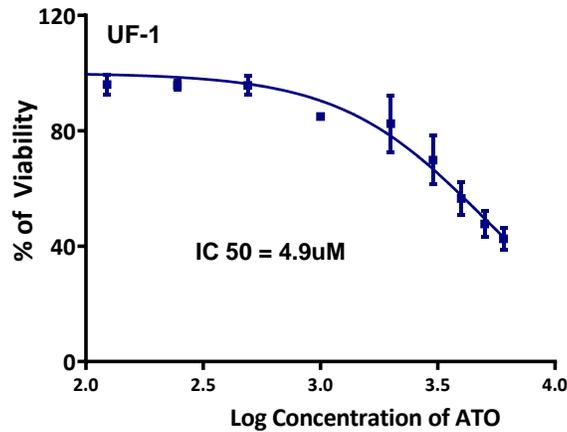
Total RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA) and transcribed into cDNA using one step cDNA synthesis kit (Applied Biosystems, MA, USA), and transcript levels were quantified by SYBR Green PCR assays with gene specific primer sets (Sigma, MO, USA). Transcript levels were calculated with the $\Delta\Delta$ Ct method, using actin as a reference gene.

Supplementary figure 1: RAR transcriptional targets in response to ATRA in resistant cell lines:



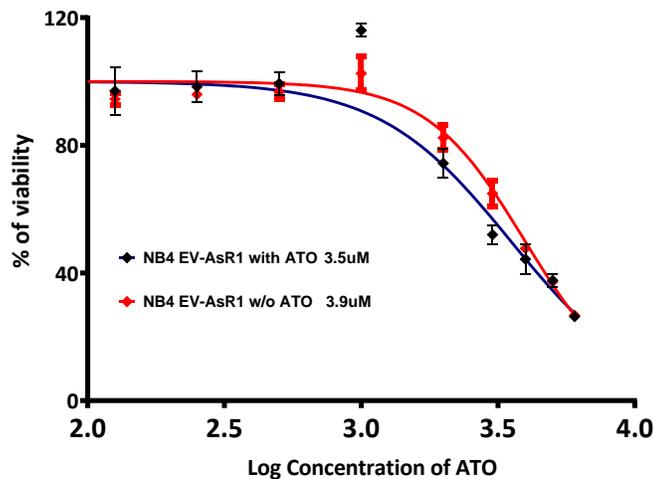
Post 24hrs exposure to ATO and ATRA as single agents and combination, relative transcript levels of RAR transcriptional targets such as TGM2 (a), RARA(b) and RARB (c) were assessed using q-PCR.

Supplementary figure 2: Half-maximal inhibitory curve (IC₅₀) of UF1, a known ATRA resistant cell line, treated with ATO for 48h hours.



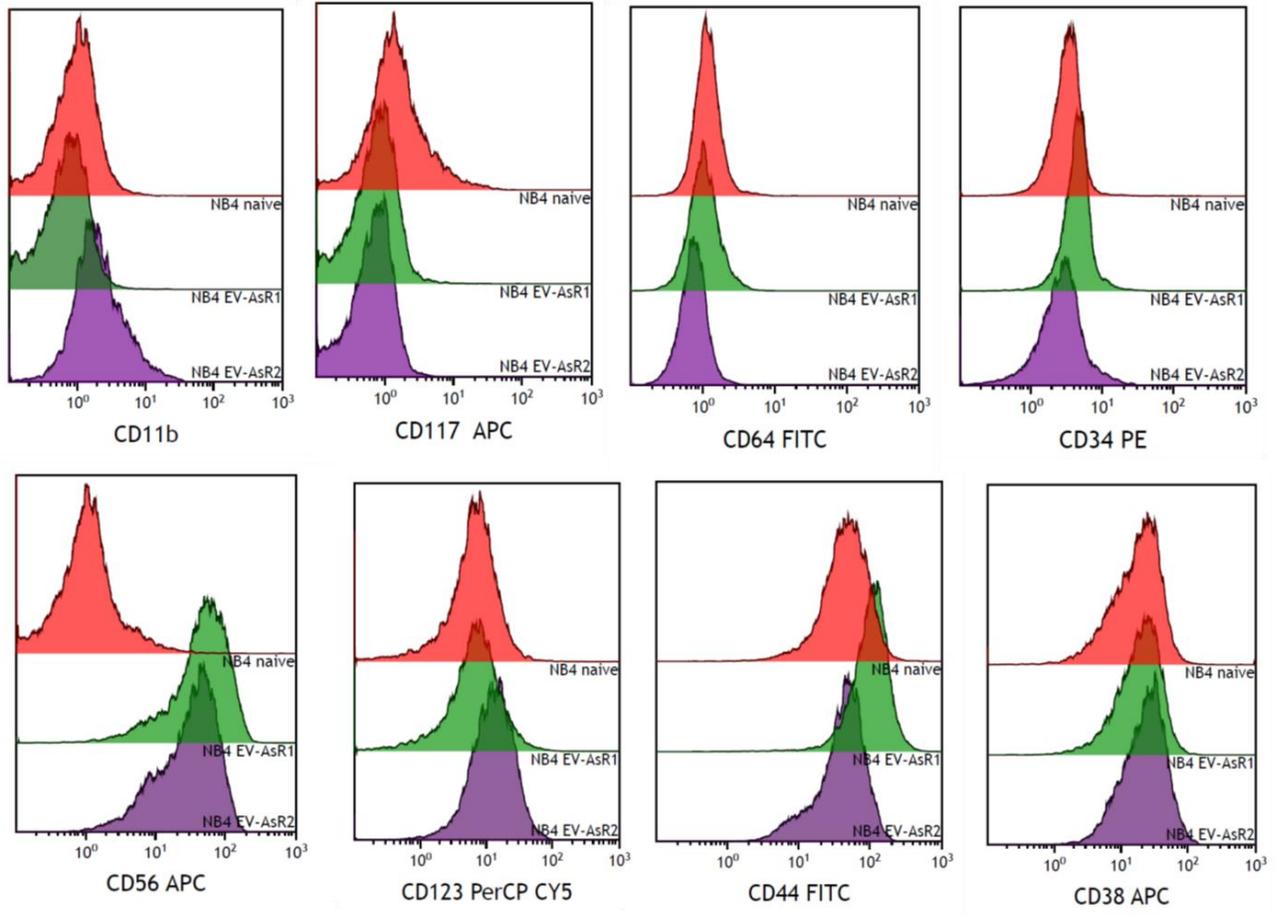
Survival curve describing the viability of UF-1 treated with the indicated concentrations of the ATO for 48 hours (n=4). All error bars represent the means \pm SEM of four independent experiments.

Supplementary figure 3: Half-maximal inhibitory curve (IC₅₀) of NB4 EV-AsR1 passaged in the presence and absence of ATO for 3 months.



Survival curves of NB4 EV-AsR1 maintained in the presence of ATO in media and without ATO (red) for 3 months and subsequent exposure to the indicated ATO concentrations for 48 hours (n=4). All error bars represent the means \pm SEM of four independent experiments.

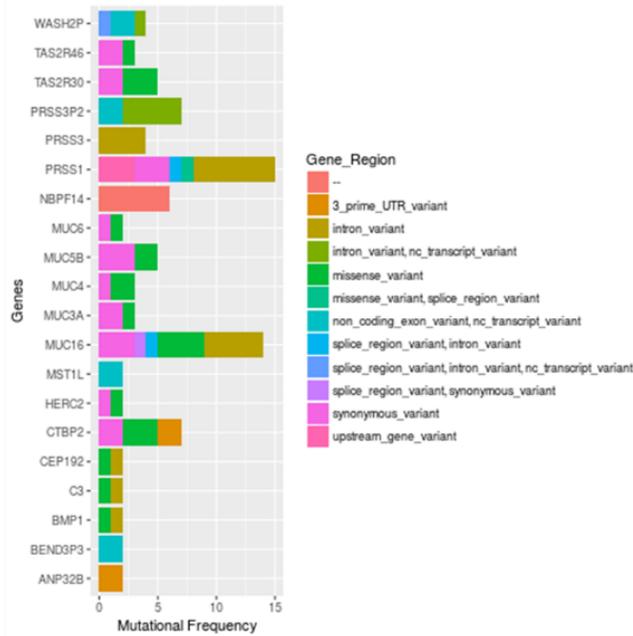
Supplementary figure 4: Surface expression of different myeloid markers in the in-house generated ATO resistant cell lines in comparison to parental NB4 naïve cells.



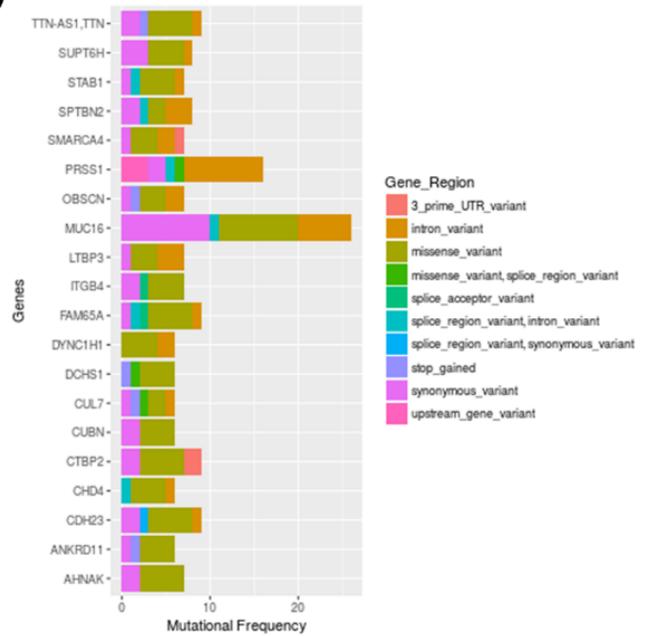
Histograms representing the immunophenotyping profile of in-house generated ATO resistant cell lines (NB4 EV-AsR1 and NB4 EV-AsR2 in comparison to parental NB4 naïve cell lines (n=4).

Supplementary figure 5: Top 20 genomic aberrations in ATO resistant cell lines.

a)

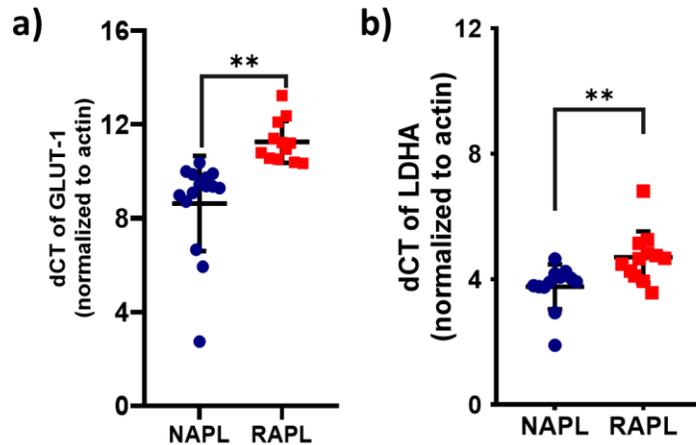


b)



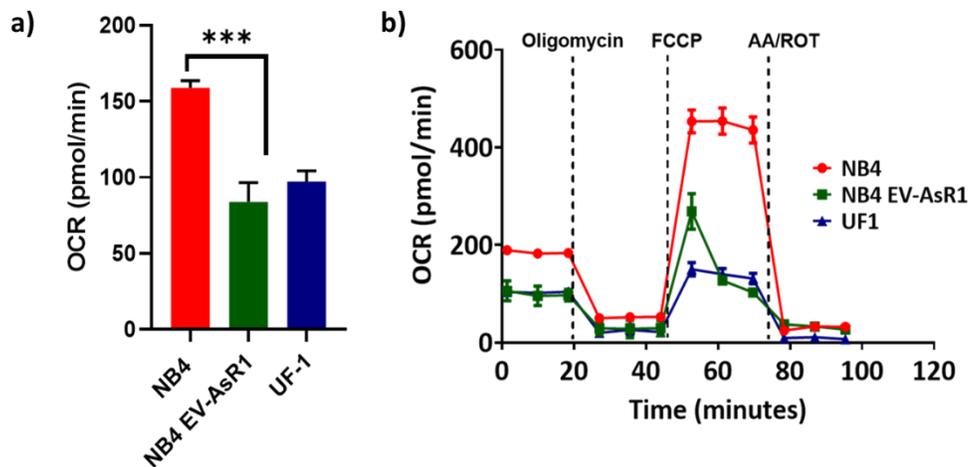
In comparison to the parental naïve NB4 cell line, top 20 mutated genes in a) NB4 EV-AsR1 and b) UF1 cell line.

Supplementary figure 6: GLUT-1 and LDHA transcript levels in the newly diagnosed and relapsed APL cells who were treated with ATO based regimen.



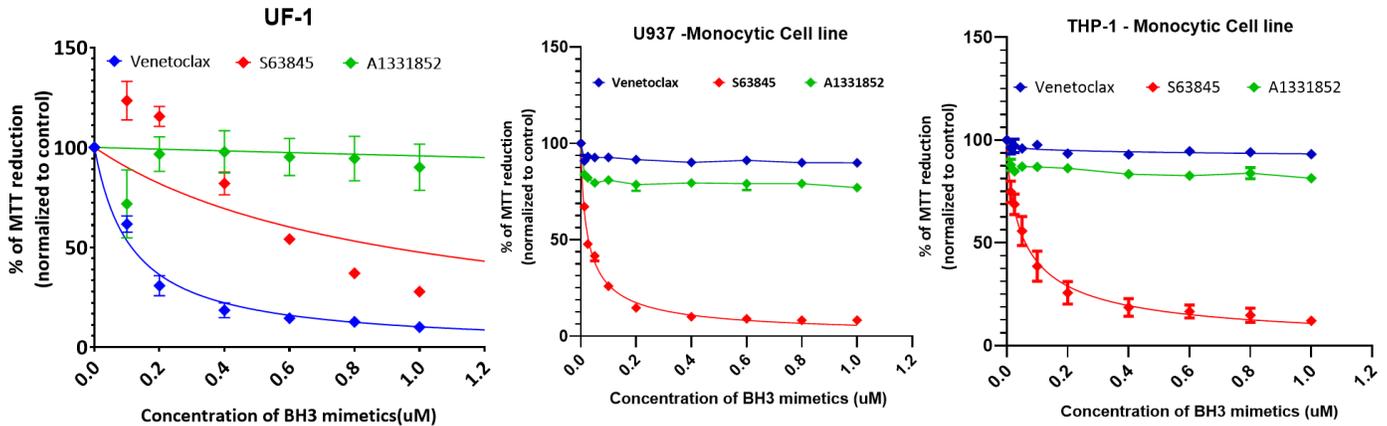
Relative expression of GLUT-1 and LDHA levels in the primary samples of newly diagnosed and relapsed APL patients who were treated with ATO based regimens (NAPL- newly diagnosed acute promyelocytic leukemia; RAPL – Relapsed acute promyelocytic leukemia) (n=12 unmatched NAPL and RAPL BM specimens)

Supplementary figure 7: Basal oxygen consumption rate (OCR) of sensitive and resistant cell lines:



a) Bar graph represents the basal oxygen consumption rate and b) Representative Mito Stress Test profile determined with a Seahorse XF analyzer

Supplementary figure 8: Half-maximal inhibitory growth curve (IC50) of APL and AML and ATO resistant cell lines treated with BH3 mimetics for 48h hours



Survival curve describing the viability of ATO resistant cell lines UF-1 (APL) and U937 and THP-1 (AML) treated with the indicated concentrations of the BH3 mimetics for 48 hours (n=3). All error bars represent the means ± SEM of 3 independent experiments.

Supplementary table 1: Modal Karyotype of NB4 Naïve and NB4 EV-AsR1

NB4 NAIVE	68~82,XX,-X,+2,+6,+7,add(7)(q36),-8,-9,-10,-11,+12,+13,+14, 15,t(15;17)(q22;q21),add(16)(24),+17,+der(17)t(15;17),-18,+18,- 19,add(19)(p13.3)x2,+20,21,+21,der(21)?i(21)(q10)x2,+22,+2~6mar[cp20]
NB4 EV-AsR1	52~62,XX,+2,+3,+4,del(5)(q14 or q14q34),+12,t(15;17)(q22;q21),add(19)(p13.3),+2~4mar[cp2]/ 68~79,XXX,+2,+4,+4,del(5)(q14 or q14q34),+6,+7,add(7)(q36),-8,-9,-10,-10,-11,+13,+14,-15,t(15;17),-18,+18,- 19,add(19)(p13.3)x2,+20,-21,-22,+22,+2~6mar[cp18]

Supplementary file 1: Mutations observed in the TCGA gene set of AML in NB4 naïve, NB4 EV-AsR1 and UF-1 cell lines.

Supplementary file 2: List of promoter regions marked with H3K27ace and H327me3 in the NB4 naïve and NB4 EV-AsR1 cell line and their gene ontology enrichment analysis using DAVID.

Supplementary 3: List of genes and their expression which was observed in newly diagnosed and relapsed APL and NB4 naïve and NB4 EV-AsR1.

The datasets discussed in this manuscript have been deposited in the NCBI under the following accession numbers

1. GSE115812 – Gene expression data of NB4 naïve, NB4 EV-AsR1 and UF-1
2. GSE42030 – Gene expression data of Newly diagnosed and relapsed APL samples.
3. PRJNA758248 - Comparison of epigenetic variations in ATO sensitive and Resistant APL cell lines.
4. PRJNA643016 - Comparing Genomic variations of ATO sensitive and Resistant cell lines.

The datasets will also be shared upon an email request to Dr.Vikram Mathews (vikram@cmcvellore.ac.in).