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Metabolic adaptation drives arsenic trioxide resistance in acute promyelocytic leukemia

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Abstract:
Acquired genetic mutations can confer resistance to arsenic trioxide (ATO) in the treatment of acute promyelocytic leukemia (APL). However, such resistance-conferring mutations are rare and do not explain most disease recurrence seen in the clinic. We have generated stable ATO-resistant promyelocytic cell lines that are also less sensitive to ATRA and the combination of ATO and ATRA compared to the sensitive cell line. Characterization of these in-house generated resistant cell lines showed significant differences in immunophenotype, drug transporter expression, anti-apoptotic protein dependence, and PML-RARA mutation. Gene expression profiling revealed prominent dysregulation of the cellular metabolic pathways in these ATO resistant APL cell lines. Glycolytic inhibition by 2-DG was sufficient and comparable to the standard of care (ATO) in targeting the sensitive APL cell line. 2-DG was also effective in the in vivo transplantable APL mouse model; however, it did not affect the ATO resistant cell lines. In contrast, the resistant cell lines were significantly affected by compounds targeting the mitochondrial respiration when combined with ATO, irrespective of the ATO resistance-conferring genetic mutations or the pattern of their anti-apoptotic protein dependency. Our data demonstrate that the addition of mitocans in combination with ATO can overcome ATO resistance. We further show that this combination has the potential in the treatment of non-M3 AML and relapsed APL. The translation of this approach in the clinic needs to be explored further.

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Agreement to Share Publication-Related Data and Data Sharing Statement: The datasets discussed in this manuscript have been deposited in the NCBI under the following accession number 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.Whole exome sequencing and Chip Sequencing data deposition has been initiated and the SRA numbers will be updated. The datasets will also be shared upon an email request to Dr.Vikram Mathews (vikram@cmcvellore.ac.in).

Clinical trial registration information (if any):

Metabolic adaptation drives arsenic trioxide resistance in acute promyelocytic leukemia

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Key points:

- Metabolic rewiring promotes ATO resistance in APL, independent of PML mutation status.
- Inhibiting mitochondrial respiration in combination with ATO can be used as a potential therapeutic option for relapsed APL and non-M3 AML.

Abstract:

Acquired genetic mutations can confer resistance to arsenic trioxide (ATO) in the treatment of acute promyelocytic leukemia (APL). However, such resistance-conferring mutations are rare and do not explain most disease recurrence seen in the clinic. We have generated stable ATO-resistant promyelocytic cell lines that are also less sensitive to ATRA and the combination of ATO and ATRA compared to the sensitive cell line. Characterization of these in-house generated resistant cell lines showed significant differences in immunophenotype, drug transporter expression, anti-apoptotic protein dependence, and PML-RARA mutation. Gene expression profiling revealed prominent dysregulation of the cellular metabolic pathways in these ATO resistant APL cell lines. Glycolytic inhibition by 2-DG was sufficient and comparable to the standard of care (ATO) in targeting the sensitive APL cell line. 2-DG was also effective in the in vivo transplantable APL mouse model; however, it did not affect the ATO resistant cell lines. In contrast, the resistant cell lines were significantly affected by compounds targeting the mitochondrial respiration when combined with ATO, irrespective of the ATO resistance-conferring genetic mutations or the pattern of their anti-apoptotic protein dependency. Our data demonstrate that the addition of mitocans in combination with ATO can overcome ATO resistance. We further show that this combination has the potential in the treatment of non-M3 AML and relapsed APL. The translation of this approach in the clinic needs to be explored further.

Introduction:

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML) characterized by the presence of reciprocal translocation between the PML gene on chromosome 15 and the retinoic acid receptor α (RARα) gene on chromosome 17 [t(15;17)], resulting in the production of a chimeric and novel PML-RARα fusion oncoprotein that leads to the differentiation block of promyelocytes to mature granulocytes[1]. Combination therapy of arsenic trioxide (ATO) and all-trans-retinoic acid (ATRA)[2-4] in the management of APL has significantly improved survival rates compared to either administered as single agents or a combination of ATRA with chemotherapy[5-7].

We recently reported on the mutational spectrum of relapsed and newly diagnosed APL patients and demonstrated the importance of additional genetic events (FLT3, KRAS, NRAS, ARID1B, p53, and WT1) during disease recurrence. However, it was also noted that mutations resulting in primary or secondary ATO resistance are extremely rare and could not explain the majority of disease relapses[8]. Other mechanisms such as bone marrow microenvironment mediated drug resistance, up-regulation of anti-apoptotic factors, modulation of cellular energy metabolism, and oxidative stress could potentially contribute to therapy resistance.

Existing literature on ATO resistance in APL has focused on the presence / acquisition of *PML* B2 domain mutations, with evidence supporting the presence of genetic mutations in the *PML* B2 domain (C212-S220; A216V) conferring resistance to ATO in APL. These mutations alter or inhibit ATO binding to the B2 domain of the PML component of the PML-RARA oncoprotein[9-13]. However, such acquired somatic mutations are rarely seen in the clinic and cannot explain the relapses that occur in patients treated with ATO based regimens. Additionally, in APL, unlike the other subtypes of AML, there is little evidence to suggest the existence of a leukemic stem cell population to explain disease recurrence[14].

Recent observations and studies report the novel mechanism of action of ATO such as promotion of non-classical apoptosis (ETosis: extracellular DNA traps) in a dose dependent manner[15] and inhibition of glycolysis. ATO directly binds to the Cys256

and Cys704 residues in hexokinase 2 (HK2) and pyruvate kinase (PKM2) reducing the enzymatic activity of these proteins and acts as glycolytic inhibitor[16]. It has further been demonstrated that this glycolytic inhibition is an important mechanism by which it promotes apoptosis in cancer cells and over expression of HK2 significantly rescued the cells from ATO induced apoptosis[16-18]. These observations further illustrate that the mechanisms of action of ATO are complex and multi-factorial, suggesting that mechanisms of resistance are also likely to be varied.

To further interrogate the mechanisms of ATO resistance we generated and characterized a stable ATO resistant cell line with the objective of finding potentially druggable targets that could be used to overcome ATO resistance in APL.

Methods:

Cell lines and chemicals:

The human APL cell line NB4 was a kind gift from Dr. Harry Iland, RPAH, Sydney, Australia, with permission from Dr. Michel Lanotte. In addition to the in house generated ATO resistant cell lines, we also used an ATRA resistant APL cell line UF1 (a kind gift from Dr. Christine Chomienne, Hôpital Saint Louis, Paris). The cell lines were free from mycoplasma contamination (Universal Mycoplasma Detection Kit, ATCC Manassas, VA, USA). Primary cells were obtained after getting written and informed consent (IRB No: 5884). Arsenic trioxide was a kind gift from INTAS Pharmaceuticals, Ahmedabad, India. 2-NBDG – fluorescent analog of D-Glucose, JC-1 and 2- Deoxy Glucose (2-DG), Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone - FCCP were purchased from Sigma Aldrich (Sigma Aldrich, St Louis, MO, USA).

Exome sequencing:

Genomic DNA was isolated from the naïve NB4 cells and the ATO resistant NB4 sub clones NB4-EVAsR1 and UF1 using Gentra puregene blood kit (Qiagen, Hilden, Germany) and stored at 4°C. Library preparation and sequencing were performed at Genotypic Technology's Genomics facility, Bengaluru, following Ion TargetSeq™

Exome Enrichment for the Ion Proton™ System, sequencing was performed on Ion Proton™ sequencer. The mutations were confirmed by Sanger sequencing.

Chromatin-immunoprecipitation sequencing (ChiP-Seq):

H3K27ace pull down was performed using simpleChIP® Enzymatic Chromatin IP Kit as per the manufacturers protocol (Cell signaling Technology, MA, USA). ChIP-Seq data was generated from two independent experimental replicates. Total genomic DNA (input) derived from formaldehyde cross-linked samples was used as control during peak calling. Raw sequence reads that passed quality control were aligned to the human reference genome (available from the UCSC genome browser, http://genome.ucsc.edu/). Peak calling on all ChIP-Seq data was performed using MACS v2.1.

Gene expression array and analysis:

A global gene expression array for differential gene expression in naïve NB4 cells and the ATO resistant NB4 primary resistant clone was performed. $2x10^7$ cells (NB4 naïve, NB4EV-AsR1, and UF1) were harvested and stored in RNA later solution. The extracted labeled RNAs were hybridized to Agilent Human Whole Genome 8x60K Gene Expression Array (AMADID: 039494), and the Image analysis was done using Agilent Feature Extraction software Version 10.5.1.1 to obtain the raw data. Normalization and statistical analysis of the microarray data were done using GeneSpring GX (Agilent Technologies, CA, USA). Differentially regulated genes were clustered using hierarchical clustering to identify significant gene expression patterns Genes were classified based on functions and pathways using biological interpretation tool Biointerpreter (Genotypic Technology, Bangalore).

Seahorse extracellular flux analysis:

Extracellular flux assay kit XF24 (Agilent Technologies, CA, USA) was used to measure oxygen consumption rate and glycolytic flux. Briefly, three replicate wells of 5×10^4 cells per well were seeded in a retronectin (Takara Bio Inc, JPY) coated 24-well XF24 plate. At 30 min before analysis, the medium was replaced with Seahorse XF

media (Agilent Technologies, CA, USA), and the plate was incubated at 37 °C. Analyses were performed both at basal conditions and after injection of glucose, oligomycin, and 2-deoxy glucose for glycolytic function.

Mouse model and drug treatments:

FVB/N mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice at 6 to 8 weeks of age were used in all the experiments. The animal study design and euthanasia protocols were approved by the institutional animal ethics committee (IAEC approval number 04/2019). APL cells from the spleen of MRP8-PML-RAR transgenic mice (FVB/N) were harvested and cryopreserved (a kind gift from Dr. Scott Kogan, UCSF, USA). APL cells (10^6 cells/mouse) were injected intravenously via the tail vein into genetically compatible FVB/N recipients, without conditioning. After the leukemic cell engraftment period (day 8), intraperitoneal injection of ATO (10mg/Kg) and 2-DG (750mg/Kg) was administered for 15 days.

Intracellular BH3 (iBH3) profiling:

10^6 cells/mL were suspended in BH3 profiling buffer, digitonin permeabilized and exposed to pro-apoptotic peptides at a concentration of 20µM (Bim, BAD, HRK and MS-1, GenScript, New Jersey, United States) for 90minutes. We then proceeded according to the standardized protocol from the Anthony Letai laboratory (http://letailab.dana-farber.org/bh3-profiling.html) to measure intracellular retention of cytochrome c. After an overnight incubation with anti-Cytochrome-c (6H2.B4)-FITC antibody (BioLegend, CA, USA) the cells were acquired on a flow cytometer (Beckman Coulter Navios, Brea, CA, USA). Loss of cytochrome-c is proportional to the priming status of the mitochondria and its anti-apoptotic dependency by their peptide specificity.

Statistical Analysis:

All statistical analyses were carried out using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). All data points are represented as means ± SEM. Two-tailed Student's t-test was used to compare mean values between two groups. For

experiments where multiple groups are compared with control, one-way ANOVA was used. P values < 0.05 were considered as statistically significant.

For further details and other methods, see supplemental methods.

Results:

Generation of arsenic trioxide resistant cell line:

ATO resistant cells were generated by exposing the naïve NB4 cell line to low concentrations of ATO (50nM) for about 3 months. The concentration of ATO was gradually increased to 1μM over one year. The cells which survived and proliferated were termed as the “ATO tolerant persister cells” (ATO-TPs). The ATO-TPs were then subjected serially to limiting dilutions and single cell colony-forming unit formation on methylcellulose to isolate monoclonal resistant populations. We isolated three different clones, expanded and named them NB4EV-AsR1, NB4EV-AsR2, NB4EV-AsR3 respectively based on the published norms of NB4 resistant cell line nomenclature[19]. The IC50 for ATO in these cell lines was 3.25μM, 3.4μM, and 2.88μM for NB4EV-AsR1, NB4EV-AsR2, and NB4EV-AsR3 respectively in contrast to naïve NB4 which was 0.9μM (figure 1a). The viability of the in-house generated ATO resistant cell lines were not significantly affected by exposure to 2μM ATO in comparison to the sensitive cell line NB4 (figure 1b). The in-house generated ATO resistant cell lines were also significantly less sensitive to differentiation-inducing agent ATRA, similar to UF1 a known ATRA resistant cell line. Even the combination of ATO and ATRA (0.5uM and 1uM respectively) did not induce a significant differentiation or cell death in the in-house generated ATO resistant cell lines when compared to naive NB4 cells (Figure 1c). Post exposure to ATRA(1uM) the induction of downstream targets of the RAR like TGM2, RARβ and RARα transcripts were also found to be significantly less in the in-house generated ATO resistant cell lines in comparison to NB4 naive (supplementary figure 1).

The doubling time of parental cell line NB4 was 28 hours, whereas for NB4EV-AsR1 and NB4EV-AsR2 clones, it was 46 and 48 hours, respectively. Long term withdrawal of ATO for 3 months from the culture system did not result in the

reacquisition of ATO sensitivity (supplementary figure 2). Currently, the resistant cell lines have a stable resistant phenotype when grown with or without ATO. We have also observed that the ATRA resistant UF1 cell line is also cross-resistant to ATO with an IC-50 of 4.9μM, an observation that has not been previously reported (Supplementary figure 3).

ATO-resistant cell lines exhibit distinct cell surface markers and transporters:

The ATO resistant cell lines were abnormal promyelocytes where the cell surface expression of the typical myeloid markers CD13 and CD33 was significantly reduced in comparison to the parental naïve cell line (figure 2a and Supplementary figure 4). The ATO efflux transporters such as AQP9, MRP4, ABCB6, and ABCA7 were significantly upregulated in the ATO resistant cell lines which mirrored in the reduced concentration of intracellular ATO (figure 2b and 2c).

Since ATO induces the degradation of PML-RARA protein, we examined the sub cellular localization, transcript, and protein levels of PML / PML-RARA in the resistant cells in comparison to sensitive cell line NB4. In the immunofluorescence assay, we observed that the PML localized in the nucleus in a typical APL specific micro-speckled pattern in the NB4 cells whereas in the resistant cell lines we observed a similar micro-speckled pattern but a decrease in the amount of nuclear PML (Figure 2d). There was also significant reduction in the levels of PML-RARA both at the transcript and protein levels in the resistant cell lines (figure 2e and 2f).

In-house generated ATO resistant cell lines harbor additional cytogenetic and molecular aberrations:

Cytogenetic analysis of both cell lines showed triploidy as well as the t(15;17). However, the NB4 EV-AsR1 showed additional cytogenetic abnormalities such as the deletion 5q, gain of chromosome 4 and loss of chromosome 22. The loss of the X chromosome as well as absence of the derivative chromosome 21 and the addition 16q which was seen in NB4 naïve was not observed in NB4 EV-AsR1 (supplementary table 1).

As there are reports implicating the emergence of drug resistance-conferring somatic PML domain mutations in APL cells against ATO, we performed whole-exome sequencing on our in-house generated ATO resistant cell line NB4EV-AsR1 (as a representative of other ATO resistant clones) in comparison to the parental cell line NB4 naïve and also on the UF1 cell line that was found to be resistant to ATO.

Whole exome sequencing revealed that in comparison to NB4 naïve, a significant number of genes were mutated in the NB4EV-AsR1 and UF1 cell lines. Based on the mutation frequency, we observed that in comparison to the NB4 naïve majority of the mutated genes in NB4EV-AsR1 belong to cell surface proteins especially mucins (MUC6, MUC5B, MUC4, MUC3A, MUC16), serine protease genes (PRSS1, PRSS3, PRSS3P2) and the ATO resistance-conferring PML B2 domain mutation (A216V). In contrast, the UF1 cell line showed a higher frequency of mutations involving MUC16, ITGB4, PRSS1, CUL7, CDH23, LTBP3, OBSCN, STAB1 genes (supplementary figure 5) and did not have a PML B2 domain mutation. Further comparison of mutated genes in the resistant cell lines with the commonly observed mutations in the AML TCGA dataset revealed that in NB4EV-AsR1 with the exception of the PML B2 domain mutation, we did not observe any additional novel mutations over and above those described in the AML TCGA gene set. However, in UF1, there were additional novel mutations found in DNMT3A, TP53, RUNX1, IDH2, SMC3, ARID1B, ARID1A, and PML genes in addition to the known mutations in the AML TCGA gene set (figure 3a and supplementary file 1).

Validation of PML and p53 mutation using sanger sequencing confirmed the existence of previously reported ATO resistance-conferring mutation A216V[10] in the in-house generated ATO resistant cell lines (including NB4EV-AsR2 and EV-AsR3). UF1 had two intronic variations in the PML domain and was negative for A216V (figure 3b). We also noted that the existence of a p53 gain of function mutation (R248Q) in the in house generated ATO resistant cell line, which was also present in the parental naïve NB4 cell line. UF1 cell line had a point mutation (R196*) and a deletion of exon 10 of the p53 (figure 3c), which are reported to be pathogenic[20].

Heterogeneity in ATO resistant cell lines:

As evident from drug withdrawal conditions and exome sequencing analysis, the observed ATO resistance was not explained by either a transient epigenetic poising or presence of a known acquired genetic mutation in the *PML-B2* domain (absence of *PML-B2* domain mutation in UF1). We next subjected NB4 naïve, NB4 EV-AsR1 and UF1 to gene expression profiling. We observed that 1717 genes in NB4 EV-AsR1 and 6149 genes in UF1 were significantly upregulated (> 2-fold) in comparison to NB4 naïve. The pathways significantly enriched for differentially expressed genes were cell survival, cell cycle, immune regulation, ABC transporters, glutathione metabolism, redox system, mitochondrial cellular respiration, and ubiquitin-proteasome degradation system (figure 4a). We also noted that the gene expression profile of the in-house generated ATO resistant cell line was similar to that of the relapsed APL patient's gene profile (8 unmatched newly diagnosed and relapsed APL) treated with front line ATO based regimens and previously reported by us[21] (Figure 4b). Gene expression profiling revealed significant dysregulation of glycolytic and mitochondrial metabolism in the resistant cell line when compared to NB4 naïve (figure 4c).

We also carried out a limited analysis of epigenetic modifications using chromatin immunoprecipitation combined with high-throughput sequencing (ChIP-Seq) for the H3k27ace, as an epigenetic marker of active enhancers and promoters and, H3k27me3 as a marker for gene repression. In the in-house generated resistant cell line NB4EV-AsR1 gene ontology enrichment on the H3k27ace mark of promoter regions showed significant enrichment for DNA damage, mitochondria, cell cycle and mRNA splicing clusters in comparison to the naïve NB4 cell line (supplementary file 2).

We validated the finding by measuring the basal metabolic properties such as reactive oxygen species (ROS), antioxidant level, glucose uptake, and mitochondrial membrane potential (MMP), which are reported to be key factors in the mechanisms of action of ATO. We observed that in comparison to naïve NB4 cells, ATO resistant cell lines (NB4EV-AsR1 and UF1) had low levels of basal ROS (Figure 5a), lower mitochondrial membrane potential (MMP) and low glucose uptake capacity (2-NBDG uptake, GLUT-1 and LDHA, both at transcript and protein level) in comparison to the naïve NB4 cells (figure 5c, d, and e). As expected, GSH levels were elevated in NB4EV-AsR1 in comparison to naïve NB4 though it remained low in UF1 (figure 5b). In patients,

consistent with the above observation in comparison to newly diagnosed primary APL samples we observed significant downregulation of GLUT-1 and LDHA transcript levels in the relapsed APL samples (supplementary figure 6).

ATO resistant APL cell lines are metabolically distinct in comparison to ATO sensitive cell line:

Glyco stress test revealed that the naïve NB4 cells had increased glycolysis where the basal extracellular acidification rate (ECAR) was significantly higher (figure 6a) with increased oxygen consumption rate (OCR) (supplementary figure 7) in comparison to ATO resistant cell lines.

To address the degree to which glycolysis is necessary for cell survival, we treated the naïve NB4 and ATO resistant cell lines with 2-Deoxy glucose (2-DG), a glucose analog that inhibits glycolysis via its action on hexokinases. We noted that naïve NB4 cell line viability was significantly affected in the presence of 2-DG, equivalent to the effect seen with 2μM of ATO. There was no evidence of an additive effect when these agents were combined (figure 6b). In contrast, the viability of the APL resistant cell lines and AML cell lines (U937 and THP-1; data not shown) was not significantly affected when 2-DG was used alone or in combination with ATO (figure 6b).

Having noted that glycolytic inhibition by 2-DG promoted apoptosis in NB4 cells comparable to that seen with ATO, we performed in-vivo glycolytic inhibition to understand the physiological relevance of glycolytic inhibition in the ATO sensitive transplantable APL mouse model. We observed that 2-DG or ATO as single agents reduced the leukemic burden in the peripheral blood (PB) and PML-RARA copy number at the end of 22 days to levels that were comparable and indistinguishable from each other (figure 6c).

Heterogeneity of anti-apoptotic protein dependency of ATO resistant APL cell lines:

We next assessed the mitochondria priming status of the ATO resistant cell lines by employing iBH3 profiling and their sensitivity to the BH3 mimetics. We observed a significant difference between the in-house ATO resistant cell lines and the parental cell line (independent of A216V). Neither a BCL-2 inhibitor (ABT-199 -Venetoclax-VEN) nor a BCL-XL inhibitor (A1331852) promoted apoptosis as a single agent in the parental and resistant cell lines whereas MCL-1 inhibitor (S63845) promoted apoptosis only in parental NB4 naïve and NB4EV-AsR2 (figure 7a). The sensitivity to the BH3 mimetics correlated with their basal BH3 profiling (figure 7b).

Combination of glycolytic inhibitor (ATO) and mitocans promoted apoptosis in the ATO resistant cell lines:

We then evaluated the effect of mitochondrial OXPHOS uncoupler on the ATO resistant promyelocytic and non-promyelocytic AML cell lines. The viability of ATO resistant cell lines was not affected significantly when treated with the mitochondrial OXPHOS un-coupler FCCP as a single agent, whereas in combination with ATO, the viability was significantly reduced in NB4EV-AsR1(harbors A216V mutation; resistant to BCL-2 and MCL-1 inhibition); NB4EV-AsR2 (harbors A216V mutation, and sensitive to MCL-1inhbition), UF1 cell line (A216V negative, sensitive to BCL-2 and MCL-1 inhibition) (Figure 7c). A similar effect was observed in non-M3 AML cell lines, U937 and THP-1 (resistant to BCL-2 inhibition and ATO whereas sensitive to MCL-1; Supplementary figure 8). We observed that the ATO resistant APL and AML cell lines, were significantly different in their metabolic preferences for their survival from the ATO sensitive NB4 naïve cell line and by targeting this difference we were able to overcome the resistance independent of the existence of the *PML-B2* domain mutation and their anti-apoptotic protein dependency. Blocking mitochondrial respiration in combination with ATO therefore can enhance cell death in ATO and ATRA resistant APL cell lines and non-M3 AML cell lines. However, this combination had significant off target effects on the normal peripheral blood mononuclear cells and further evaluation for compounds that selectively target the leukemic cell's mitochondrial respiration is needed for these observations to be translated to the clinic.

Discussion:

The study highlights that in comparison to the existing ATO resistant APL cell lines[22,23] our in-house generated ATO resistant cell lines are stable, well-characterized at the genomic levels and they also possess the well-known ATO resistance-conferring mutation A216V in the B2 domain of the PML-RARA oncoprotein. These ATO resistant cell lines are observed to be less sensitive to differentiation with ATRA and to the combination therapy of ATO and ATRA and are hence an excellent tool to evaluate additional mechanisms that could contribute to drug resistance. We also noted that the UF1 APL cell line was resistant to ATO (not been previously reported) but did not have the well-defined PML-RARA B2 domain mutation giving us an opportunity to study ATO resistance with and without PML-RARA B2 domain mutations.

In comparison to the naïve NB4, in house generated ATO resistant cell lines overexpressed ATO efflux transporters such as AQP9, MRP4, and ABCA7, which correlated with their inability to accumulate intracellular ATO. This phenomenon was not observed in primary blasts from relapsed APL patients previously treated with ATO in comparison to newly diagnosed[21]. The possibility of efflux transporters as a protective mechanism of ATO resistance cannot be excluded in the clinic and needs further evaluation.

We noted the presence of a somatic mutation in the B2 domain of the *PML* gene in our in-house generated ATO resistant cell line that has been reported to confer resistance to ATO. It is important to note that these mutations are acquired post ATO treatment, and none of the APL cell lines or relapsed APL patients prior to ATO therapy had the B2 domain mutation[24]. The in-house resistant cell lines did not acquire additional mutations in p53 gene which has been reported to be critical for the regulation of NB formation and ROS generation. The observation suggests that the reduction in the PML micro-speckled pattern formation and low ROS levels are independent of p53 status in these cell lines. The cellular redox system of the resistant cell lines was found to be significantly altered in these cell lines having lower ROS levels, lower proliferative rate, and an increased antioxidant system favoring quiescence

and stemness like properties[25]. This was further corroborated by the observation of low ECAR and OCR status of the resistant cell lines [26].

ATO sensitive cell line NB4 naïve was observed to be more reliant on the Warburg effect for their survival and proliferation, and their viability was significantly affected by a glycolytic inhibitor (2-DG). In an in-vivo APL model, 2-DG significantly reduced the leukemic burden comparable to the standard of care (ATO). This further supports our observation that the naïve ATO sensitive APL cells survival could be targeted by glycolytic inhibition. Based on the recognized inhibitory effect of ATO on the glycolytic pathway[17] this could also be an important mechanism by which ATO induces apoptosis in malignant promyelocytes. In contrast, the ATO resistant APL and AML cell lines were mainly dependent on OXPHOS for their survival. However, unlike NB4 naïve cells the ATO resistant and AML cell lines had greater metabolic plasticity to switch between glycolysis and the OXPHOS when one is inhibited but were susceptible to a combination of ATO and mitocans, this susceptibility was independent of their PML B2 domain mutation status and their anti-apoptotic protein dependency. It is well known that A216V mutation alters the ATO binding on the B2 domain of the PML-RARA oncoprotein, the observed combinatorial effect of ATO in combination with mitocan on the resistant cell lines is hence likely to be due to the inhibitory effect of ATO on the glycolytic pathway[13,27].

Taken together, our work demonstrates that ATO resistance is multi-factorial and is not limited to presence or absence of either PML or p53 mutations. The in-house generated ATO resistant cell line would be a useful model to further evaluate mechanisms of resistance in leukemia. Targeting the metabolic adaptations seen in ATO resistant cell lines has the potential to overcome such resistance. Inhibiting mitochondrial respiration in combination with ATO could overcome ATO resistance and translating this approach to the clinic both in relapsed APL and in the treatment of newly diagnosed AML needs to be explored further. While the combination of ATO and FCCP was observed to be non-selective, there are a number of FDA approved drugs used in the clinic that have the property to selectively inhibit mitochondrial respiration in cancer cells, these could potentially be evaluated for their synergistic activity with ATO in

leukemia[28]. Our data also draws attention to possible severe off target toxicity of such combinations which may be inadvertently used in the clinic.

Data Sharing Statement:

The datasets discussed in this manuscript have been deposited in the NCBI under the following accession numbers: 1) GSE115812 [GEO] - Gene expression data of NB4 naïve, NB4 EV-AsR1 and UF-1. 2) GSE42030 [GEO] - Gene expression data of Newly diagnosed and relapsed APL samples. 3) PRJNA758248 [BioProject] - Comparison of epigenetic variations in ATO sensitive and Resistant APL cell lines. 4) PRJNA643016 [BioProject] - 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).

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Author contribution:

NB: performed research, involved in designing the study, performed molecular tests, and analyzed data and written the paper.

SG: performed research, performed molecular tests, and analyzed data.

EC: performed research, performed molecular tests, and analyzed data.

HKP: performed research, performed molecular tests, and analyzed data

AV: performed research, performed molecular tests, and analyzed data

AAA: performed research, performed molecular tests, and analyzed data.

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SPK: performed research, performed molecular tests, and analyzed data.

NRR: performed research, performed molecular tests, and analyzed data.

MY: performed research, performed karyotyping tests, and analyzed data.

AK: performed research and analyzed data.

UK: performed research and analyzed data.

NBJ: performed research, performed karyotyping tests, and analyzed data.

SK: performed research and analyzed data.

PB: performed research and analyzed data.

VM: performed research, designed study, analyzed data, and written the paper.

Conflict of interest: No conflict of interest

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Figure Legends:

Figure 1: Generation of ATO resistant cell lines. a) NB4 naïve parental cell line was exposed to 50nM of ATO for three months, and the concentration was gradually increased to 1µM ATO over a period of one year until they sustained and proliferated. Limiting dilutions and colony-forming unit assay were performed to generate mono clones of the resistant cell lines. b) The bar graph represents the percentage of viable cells post 48 hours of 2µM ATO. c) Representative dot plots and stacked bars (summarizes the dot plots results) NB4 and resistant cell lines were treated with 0.5µM and 1uM of ATRA for 72hrs as single agents and in combination with 2uM ATO, and the percentage of differentiation was measured by the surface expression of CD11b and dead cells were measured by 7AAD. Graphs and statistical parameters were generated from three independent experiments. *p≤0.05; **p≤0.01; ***p≤0.001; ***p≤0.0001.

Figure 2: Heterogeneity in the cell surface marker and transporters expression of the ATO resistant cell line in comparison to parental cell line: a) CD13 and CD33 surface expression of ATO resistant cell lines in comparison to NB4 naïve. b) Relative mRNA levels of ATO influx gene (ABCA1) and efflux transporters genes (ABCA7, AQP9, MRP4, and ABCB6) in the in-house generated ATO resistant cell lines compared to NB4 naïve which is normalized to one. c) Intracellular ATO levels in NB4 naïve and ATO resistant cell lines post 24 hours of 0.5µM ATO treatment. d)Fluorescent microscopic image of the PML (RED) in NB4 naïve and ATO resistant cell lines displaying nuclear body formation and micro speckled pattern (magnification 63x – oil immersion) e) PML-RARA transcripts in the NB4 naïve and in-house generated ATO resistant cell lines f) Immunoblots of the PML-RARA fusion protein levels in NB4 naïve and ATO resistant cell lines. All error bars represent the mean ± SEM of three independent experiments. *p≤0.05; **p≤0.01; ***p≤0.001; ***p≤0.0001.

Figure 3: Whole-exome sequencing reveals changes in the ATO resistant cell lines at the genomic level. a) AML TCGA data set was compared with novel mutations observed

in AsR1 and UF1, and the graph represents the novel mutations (only found in resistant cell lines) and their mutation frequency. B) Sanger sequencing showing the existence of PML – A216V in the in-house generated ATO resistant cell line and not in the UF1 and parental cell line NB4 naïve. c) Mutations observed in the p53 gene of ATO resistant and sensitive cell lines.

Figure 4: Gene expression analysis of the ATO resistant cell lines reveals dysregulation of cellular metabolism. a) Pie chart representing the dysregulated genes in the in-house generated ATO resistant cell line NB4 EV-AsR1 and UF1 in comparison to parental cell line NB4 naïve. b) Heatmap highlighting gene signature of NB4 naïve (duplicate) in-house generated ATO resistant cell line (duplicate) and the 8 unmatched newly diagnosed and relapsed APL samples (primary cells data previously reported and adapted from Ezhilarasi Chendamari., et.al., PloS one 2015). c) Heatmap representing NB4 naïve, ATO resistant cell line NB4 EV-AsR1 and UF1 cell lines genes involved in the glycolytic and mitochondrial metabolism.

Figure 5: ATO resistant cell lines are metabolically distinct. a) Baseline total reactive oxygen species were measured using redox-sensitive dye (cell ROX Green) by flow cytometry. b) Baseline protein thiols was measured as an indicative of antioxidant using OPT (Phthaldialdehyde) and median fluorescence intensity are represented as bar graphs. c) Mitochondria membrane potential of the resistant cell lines were measured using JC-1 d) Glucose uptake was measured using a fluorescent analogue of 2- deoxy glucose and represented as relative mean fluorescence intensity. GLUT-1 and LDHA transcripts (e) and protein (f) levels of NB4 naïve, NB4 EV-AsR1 and UF1 cell lines. All error bars represent the means ± SEM of three to four independent experiments. *p≤0.05; **p≤0.01; ***p≤0.001; ***p≤0.0001.

Figure 6: ATO resistant cells are metabolically heterogeneous and *in vivo* effect of glycolytic inhibition by 2-DG reduces leukemic burden in APL mouse model**.** a)

extracellular acidification rate (ECAR) and glycolytic potential of NB4 naïve, NB4 EV-AsR1 and UF1 cell lines were assessed in real time using seahorse extracellular flux analyzer. b) Viability of the sensitive and resistant cell lines post 48 hours of glycolytic inhibitor ATO and 2-DG (ATO = 2µM; 2-DG = 5mM; 48 hours; n=4). All error bars represent the means ± SEM of four independent experiments. *p≤0.05; **p≤0.01; ***p≤0.001; ***p≤0.0001. c) Schematic representation of the APL transplantable mouse model and treatment plan. Mice were euthanized on day 22 and examined for the presence of leukemic cells as CD117+Gr1+ cells in peripheral blood, and PML-RARA transcript levels in bone marrow.

Figure 7: Mitocans synergize with ATO to promote apoptosis in the ATO resistant cell lines. a) Viability of NB4 and in-house generated ATO resistant cell lines to BH3 mimetics (n=5). b) Intracellular BH3 profiling of ATO resistant and sensitive cells (n=3) measured using intracellular cytochrome-c retention. c) Viability of the sensitive and resistant APL and non-APL cell lines treated with OXPHOS uncoupler FCCP in combination with ATO. ATO = 2µM; FCCP =10µM and BH3 mimetics = 250nM; 48 hours. All error bars represent the means ± SEM of four independent experiments. *p≤0.05; **p≤0.01; ***p≤0.001; ***p≤0.0001. (ABT-199 – Venetoclax; S63845 - MCL-1 inhibitor; A1331852 – BCL-XL inhibitor; ALM – Alamethicin).

Figure 1

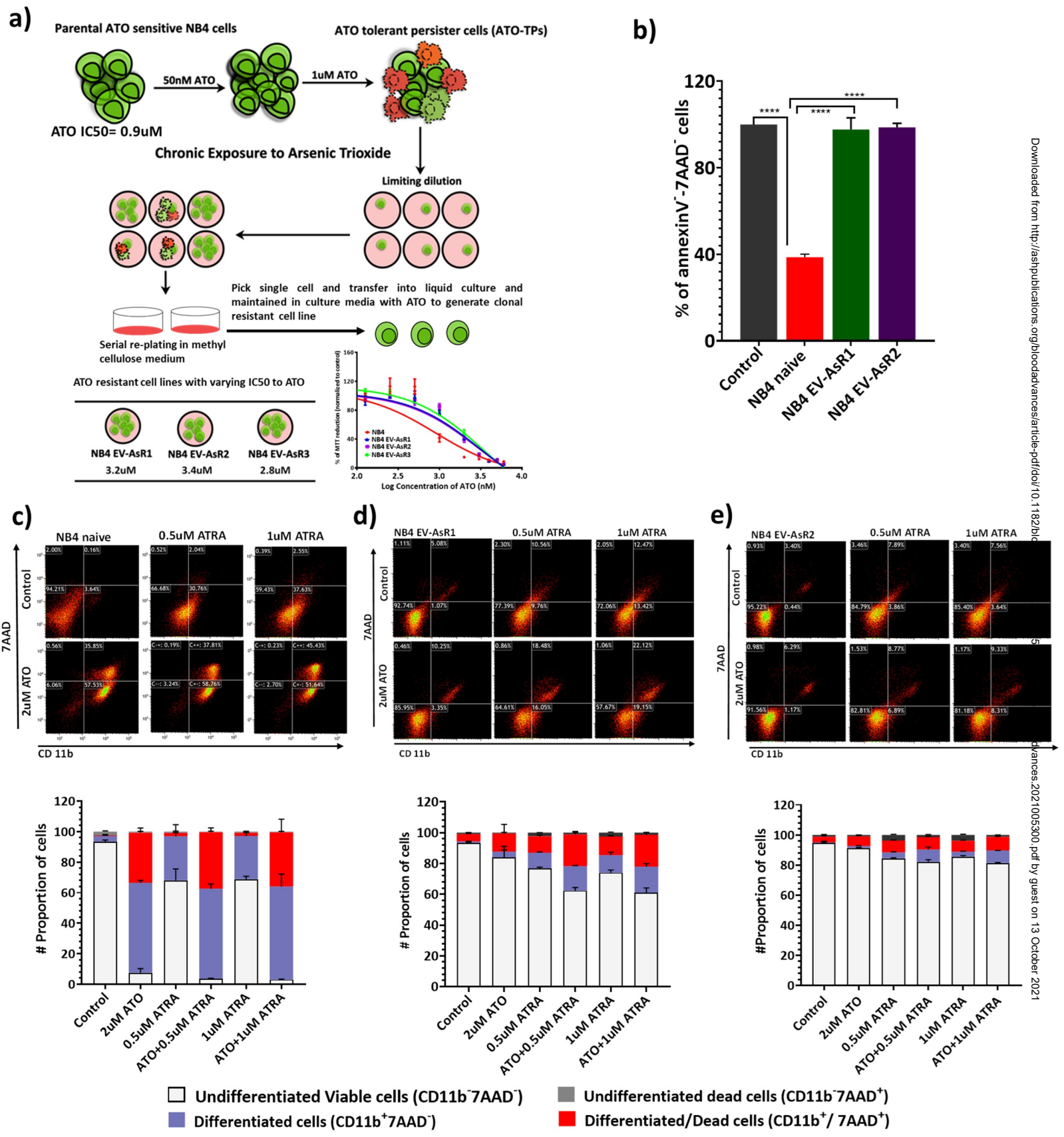


Figure 2

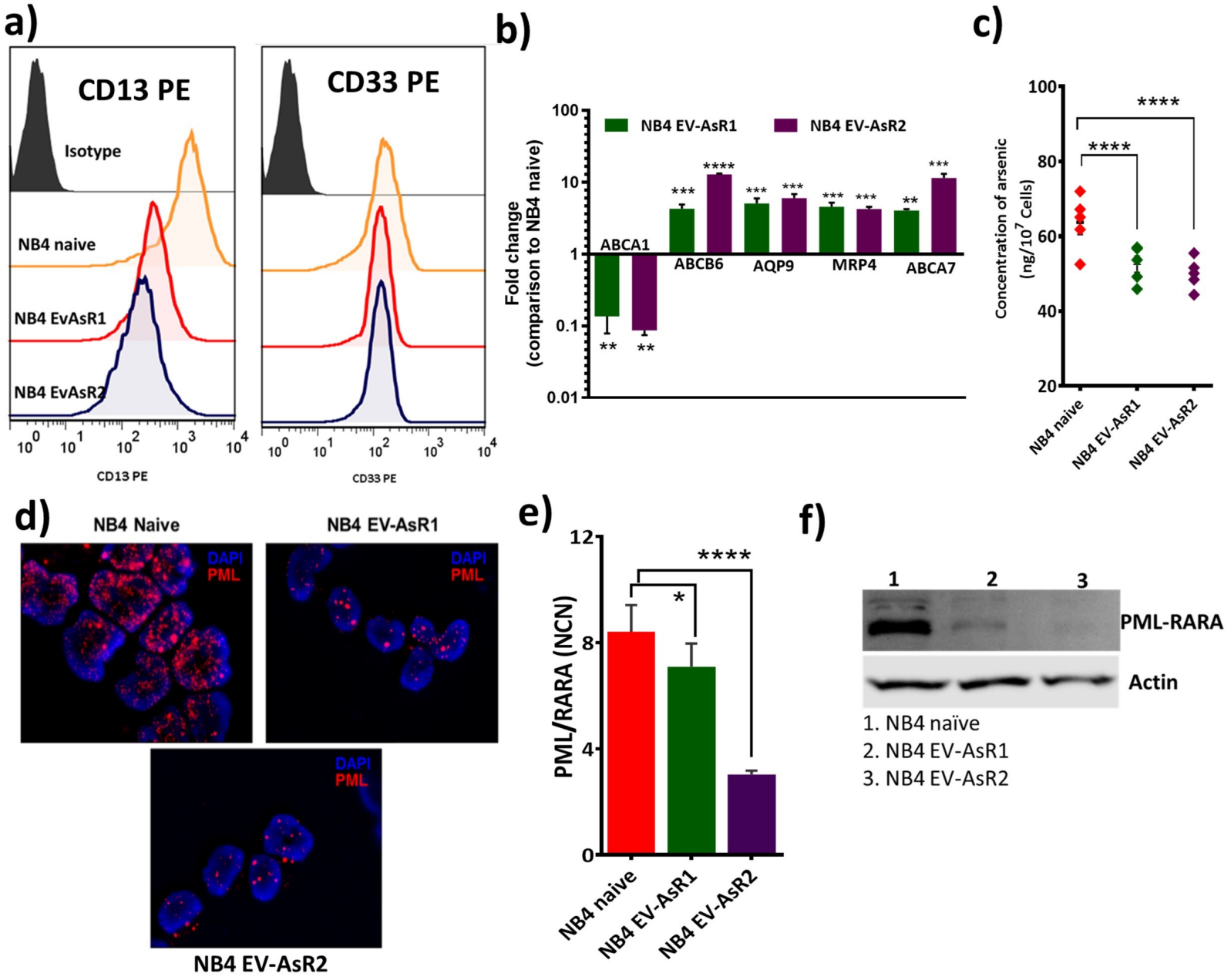
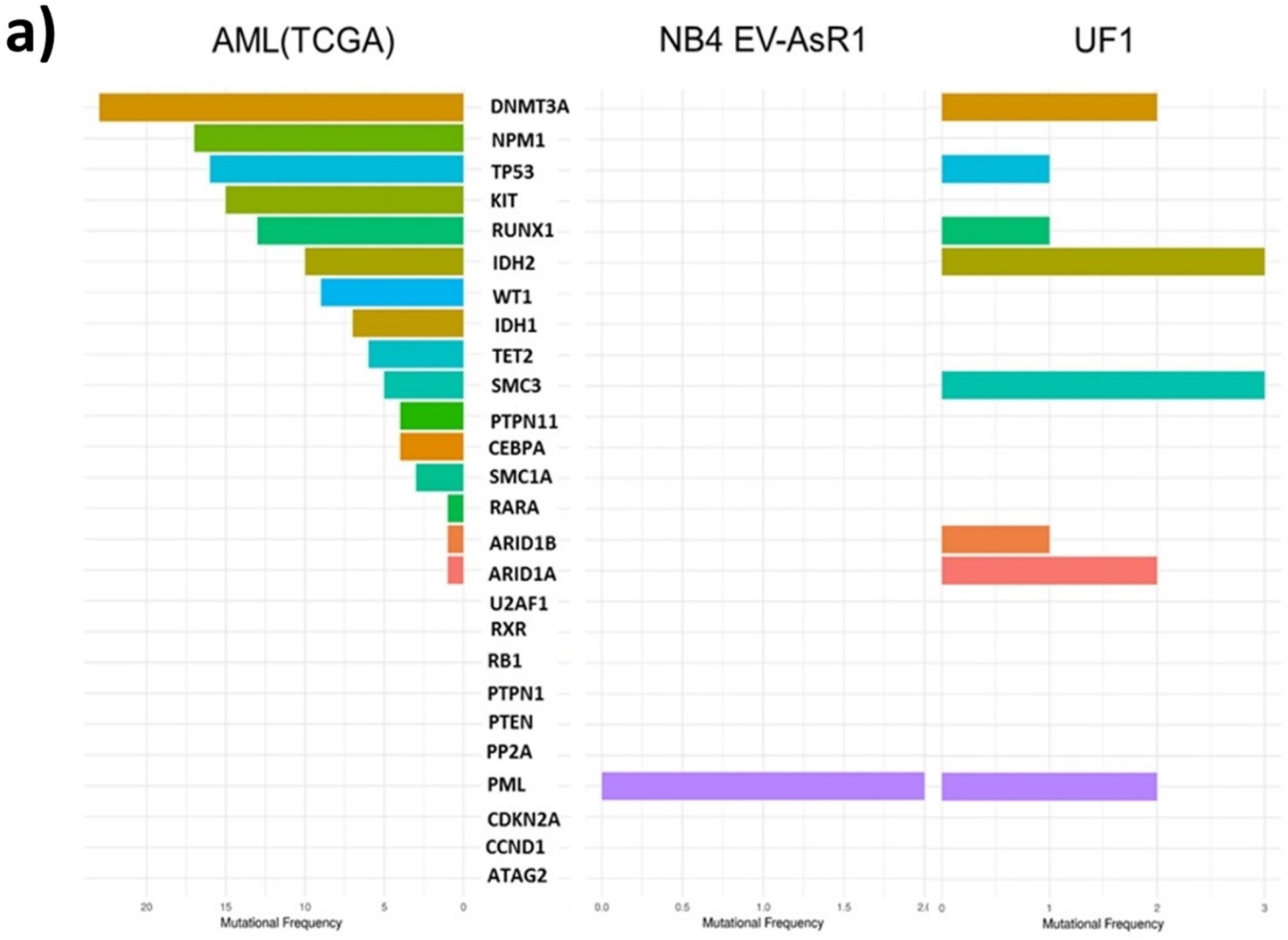
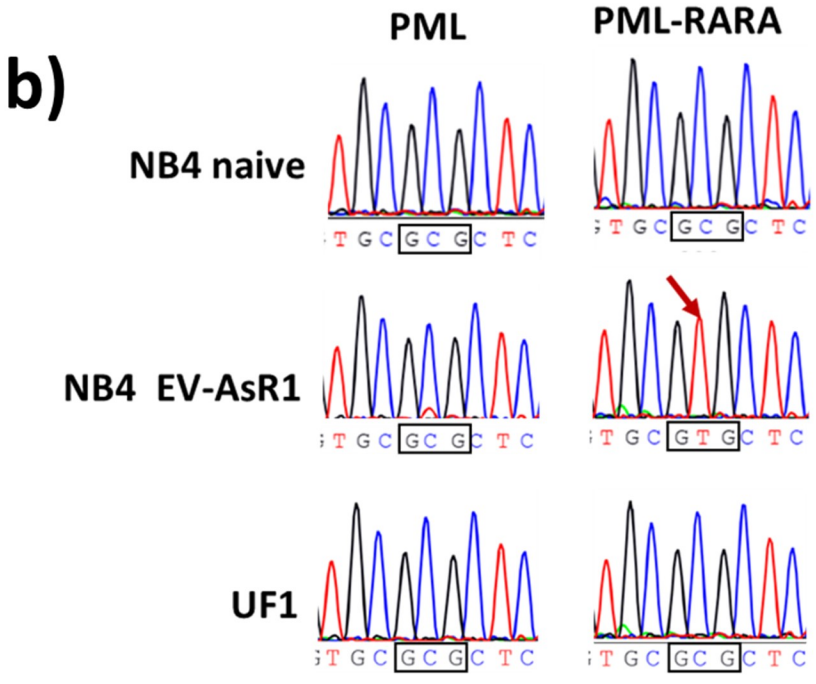


Figure 3





c)

Cell line	Exon	TP53 mutation
NB4 naïve	7	c.743 G>A (R248Q)
NB4 EV-AsR1	7	c. 743 G>A (R248Q)
UF1	5	c.586 C>T (R196*)
	10	c.1083_1083 del (S362Afs*8)

Figure 4

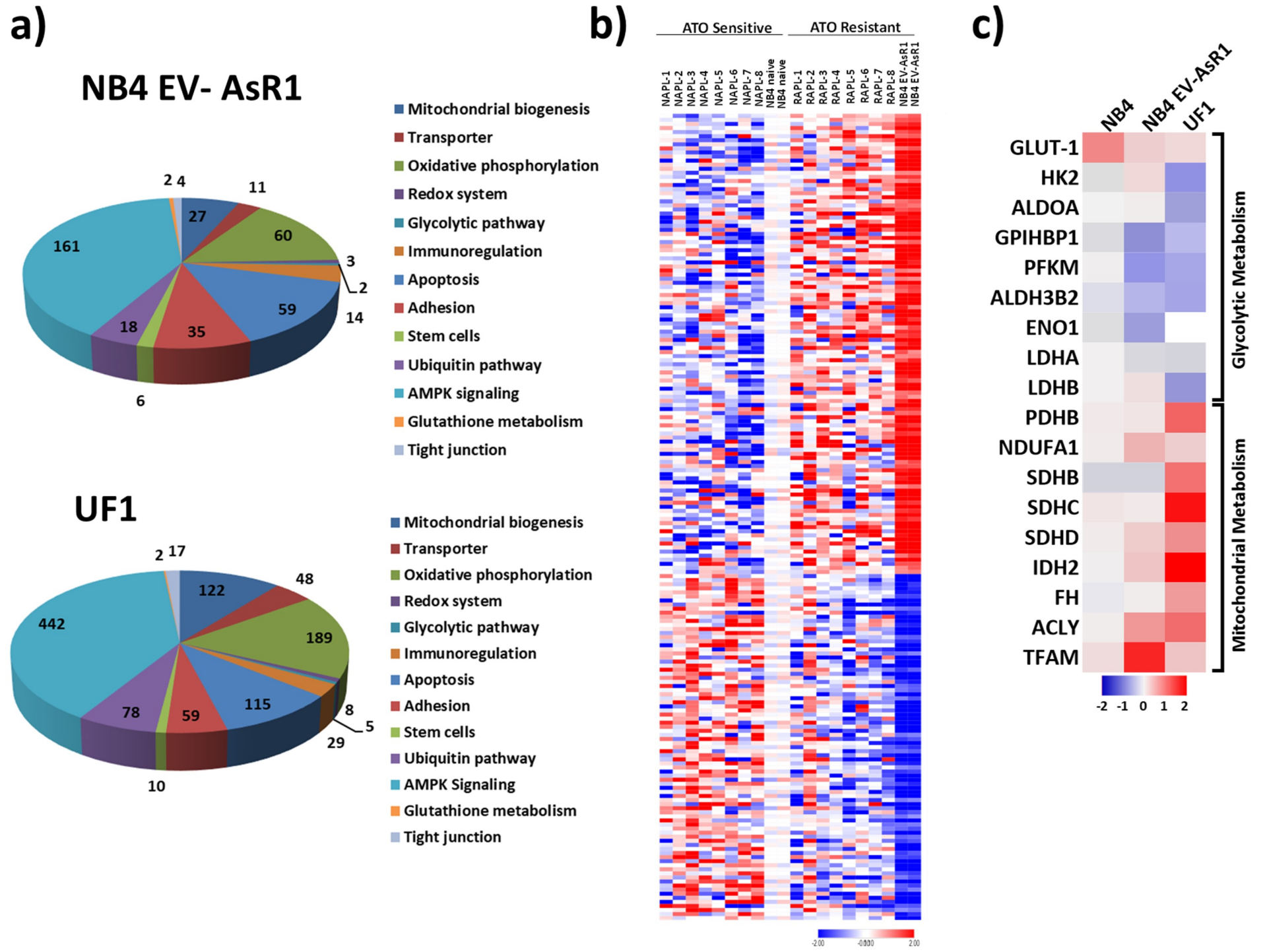


Figure 5

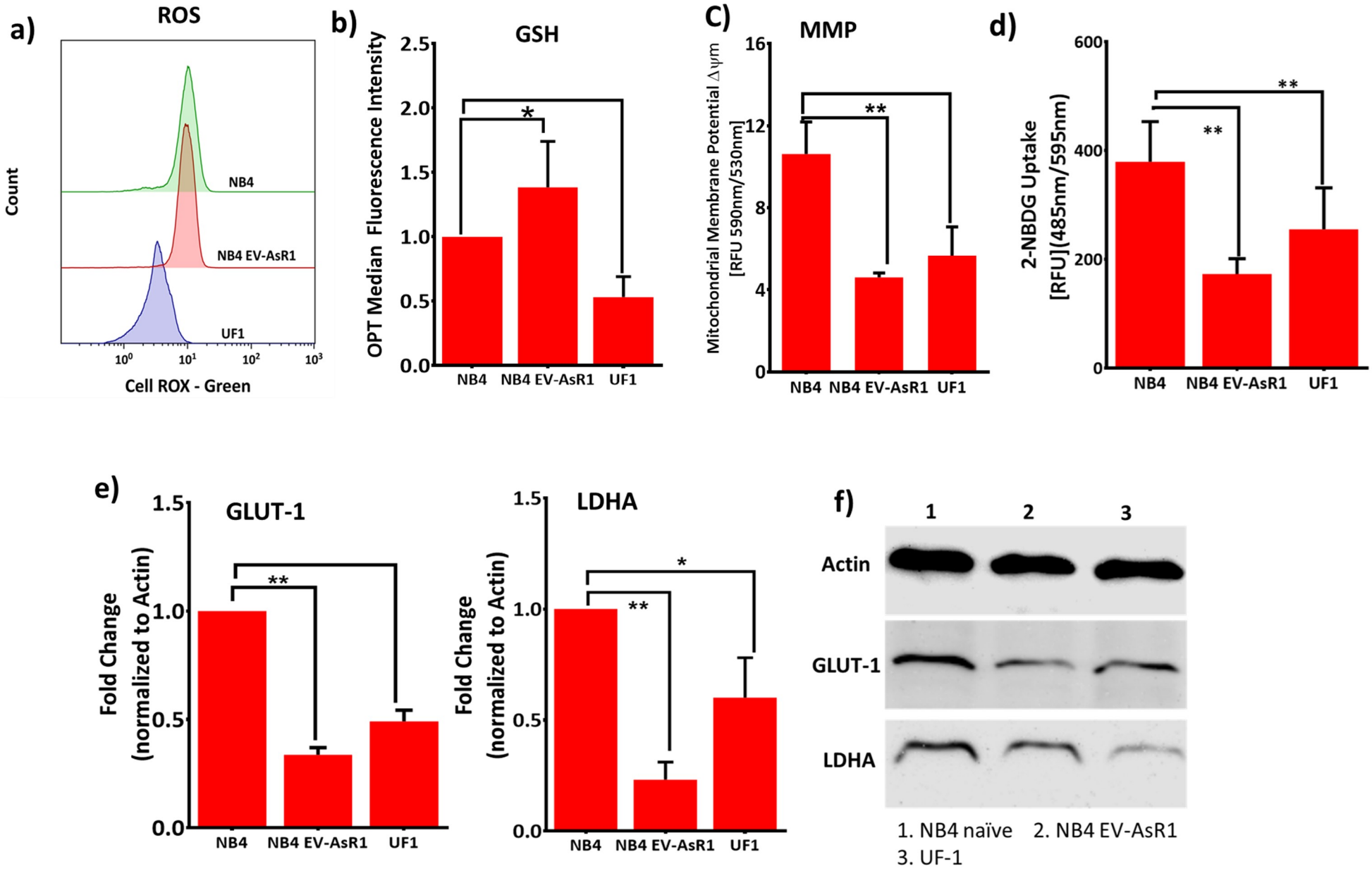


Figure 6

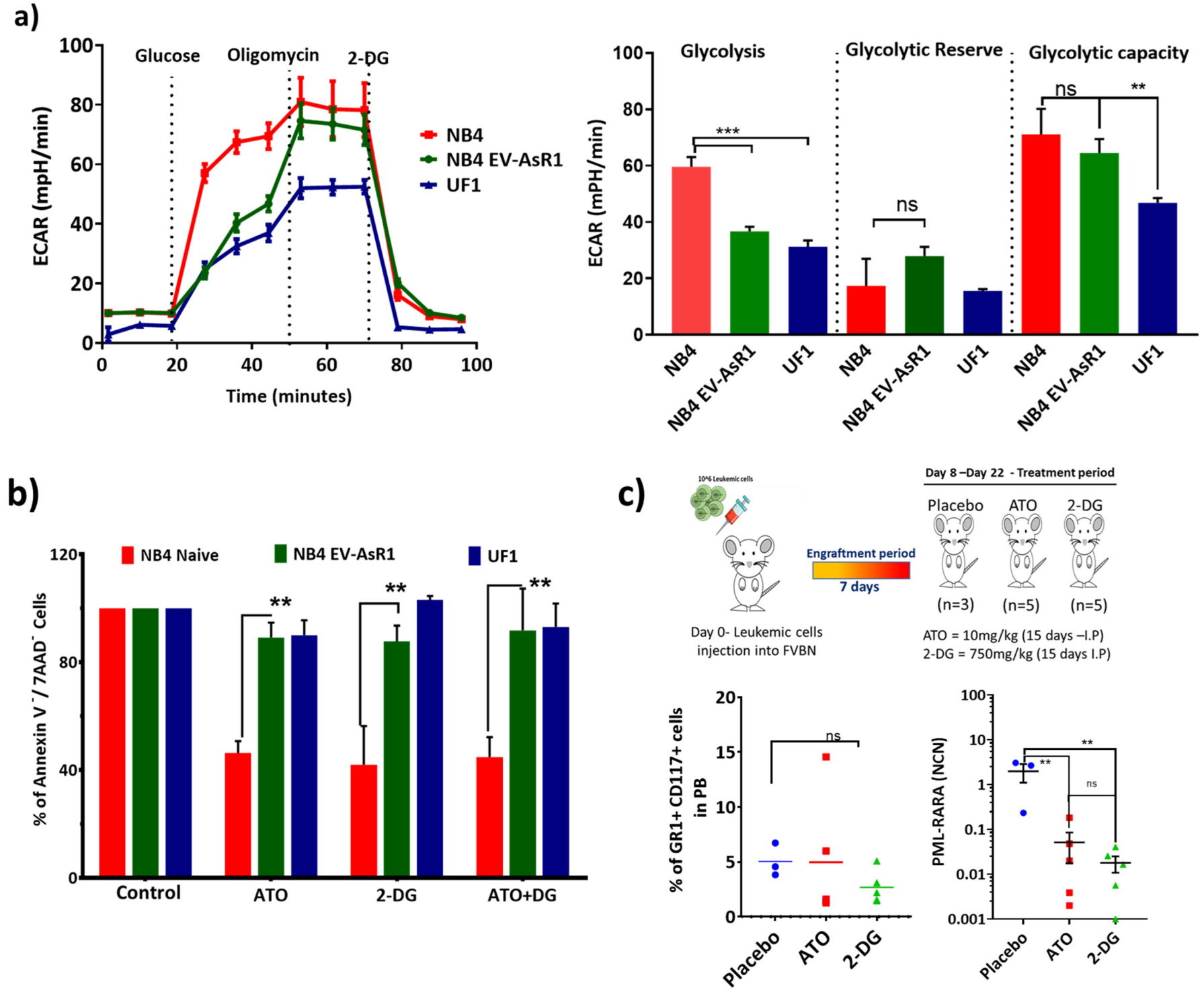


Figure 7