Chemotherapy Resistant Human Acute Myeloid Leukemia Cells are Not Enriched for Leukemic Stem Cells but Require Oxidative Metabolism


Supplementary Methods

Reagents

Cytarabine (AraC), idarubicin (IDA), and etoposide (VP16) were provided by the Pharmacy of Toulouse University Hospital (Toulouse, France). Phenformin hydrochloride (Phenf), rotenone (Rot), atovaquone (ATQ), antimycin A (AntiA), tigecycline (Tig), 1,1-dimethylbiguanide hydrochloride (Met), oligomycin (oligo), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), sodium iodoacetate (ido) and busulfan were obtained from Sigma-Aldrich. (R)-(+) Etomoxir was supplied by Medchem Express.

Cytarabine treatment for Cell Lines Derived Xenograft

As described for PDXs and in Saland et al. (26), NSG mice were sublethally treated with busulfan (20 mg/kg/d) 24hr before injection of leukemic cells. Cell Lines-Derived Xenograft (CLDX) were generated by IV injection of AML cell lines at a final concentration of 2 million cells per 200 µL of Hanks Balanced Salt Solution per mouse. 10 days after cell transplantation, NSG mice were treated by daily intraperitoneal (IP) injection of 30 mg/kg AraC for 5 days. For control, NSG mice were treated daily with IP injection of 200 µL of PBS as vehicle. Bone marrow and spleen were harvested at day 8 after the first dose of AraC.

Cell cycle and immunophenotype analysis

After dissection, bone marrow samples were immunophenotyped and used for cell cycle analysis. For quantification of cells in G0 phase of cell cycle, AML-engrafted recipient bone-marrow cells were labeled with IV injection of AML cell lines at a final concentration of 2 million cells per 200 µL of Hanks Balanced Salt Solution per mouse. 10 days after cell transplantation, NSG mice were treated by daily intraperitoneal (IP) injection of 30 mg/kg AraC for 5 days. For control, NSG mice were treated daily with IP injection of 200 µL of PBS as vehicle. Bone marrow and spleen were harvested at day 8 after the first dose of AraC.

Secondary transplantation with limiting dilution analysis

Adult recipient mice (6-8 weeks old) were sublethally irradiated with 225cGy total body irradiation 24 hours prior to secondary transplantation. Engrafted BM samples from AML-xenografted mice (for 4 primary AML specimens) untreated (Vehicle) or treated with AraC were lysed with 0.8% ammonium chloride solution (StemCell Technologies), washed twice in PBS containing 2% FBS, and suspended in 200 µL PBS containing 2% FBS. Specific cell doses of human viable cells from BM of primary recipients per mouse, ranging from 50 cells to 3.10^5 cells were injected IV into at least 3 NSG mice per dose. 12 to 18 weeks after transplantation, BM and spleen were assessed for the engraftment. An engraftment criterion of more than 0.1% of human CD45+CD33+ in murine BM mononuclear cells assessed by flow cytometry was used as the biologically significant cutoff. The frequency of LICs was calculated using Poisson statistics with the L-Calc Software for limiting dilution analysis (StemSoft Software version 1.1; StemCell Technologies).

Histological analysis of NSG mice bone sections

Paraformaldehyde-fixed, decalcified, paraffin-embedded sections were prepared from femurs, tibias and sternums of the recipients transplanted with primary AML specimens. Goldner staining was performed using standard procedures by the Histology Core at CPTP INSERM. Light microscopy was performed using Leica 400 (Leica).
**Pimonidazole binding and imaging analysis to assess redox status of AML residual cells in vivo**

The detection of intracellular redox in BM was performed by PIMO binding using a modified intracellular staining. AML-xenografted mice were injected intraperitoneally with Hypoxyprobe™-1 (Chemicon) at 60 mg/kg dose and BM were harvested after 1h post-injection. Paraformaldehyde-fixed (PFA), decalcified, paraffin-embedded sections were prepared from femurs by the Histology Core at CPTP INSERM. Then, sections were stained for pimonidazole using the FITC-conjugated antipimonidazole antibody (1:1,000 dilution), and were counterstained with DAPI to visualize nuclear DNA and PE-conjugated anti-human CD33 antibody (1:1,000 dilution, Novocastra Laboratories) to visualize human leukemic cells. Immunofluorescent images were obtained with a fluorescence microscope and objective coupled with a digital camera.

Alternatively, flow cytometry analysis of pimonidazole staining has been done as described for immunohistological imaging. Briefly, AML-xenografted mice were injected intraperitoneally with Hypoxyprobe™-1 (Chemicon) at 60 mg/kg dose and BM were harvested and dissected after 1h post-injection. Cells were stained with CD33-PE and CD45-V450 conjugated antibodies (BD) and then fixed with 1% PFA solution for 10 min and permeabilized with a 0.02% saponin solution again for 10 min. FITC-conjugated anti-pimonidazole antibody (1:100 dilution) was added for 30 min at 37°C and finally processed by flow cytometry.

**Measurement of ROS content, mitochondrial membrane potential and mitochondrial mass**

Total ROS content, mitochondrial membrane potential and mitochondrial mass in viable human CD45^+CD33^+ blasts were measured by flow cytometry using DCF-DA, TMRE and MitoTracker Green (MTG) probes, respectively (Invitrogen).

**FACS-based cell sorting**

Human AraC-residual AML cells from AraC-treated AML-xenografted mice or AraC-treated AML cell lines in vitro were stained with 7-AAD, V-500 conjugated-AnnexinV and the following fluorescently-conjugated antibodies: APC-H7-conjugated anti-hCD45, PercP-conjugated anti-mCD45, PE-Cy7-conjugated anti-hCD44, PE-conjugated anti-hCD33 and DCF-DA probe. Cells were sorted into two subsets of Low and High ROS (DCF-stained) cells by MoFlo Astrios (Beckman Coulter).

**EC₅₀ experiments**

Sorted and unsorted AraC residual cells were treated for 24 hours. To measure half maximal inhibitory concentration (EC₅₀), increased concentrations of AraC, idarubicin and VP-16 were added to the medium. The doses that induce apoptosis to 50% (EC₅₀) were analyzed by the median-effect method with GraphPad Prism software.

**Measurement of oxygen consumption in AML cultured cells**

**Clark Electrode Assay.** AML cell lines were treated with AraC, mitochondrial inhibitors or vehicle for 24h in MEMa media. Following treatment, 500,000 cells were resuspended in 100 μL of fresh medium and analyzed in a water-jacketed, air-tight chamber with a Strathkelvin Clark oxygen electrode. Measurements were taken at 37°C with constant stirring. The specific oxygen consumption rates represent the following measurements: basal rate, ATP turnover (basal rate minus oligomycin-insensitive rate i.e. oligomycin sensitive), proton leak (oligomycin-insensitive rate), maximal respiration (FCCP/uncoupler-stimulated rate) and spare respiratory capacity (uncoupler-stimulated minus basal rate).

**Seahorse Assay** All XF assays were performed using the XFp Extracellular Flux Analyser (Seahorse Bioscience, North Billerica, MA). The day before the assay, the sensor cartridge was placed into the calibration buffer medium supplied by Seahorse Biosciences to hydrate overnight. Seahorse XFp microplates wells were coated with 50 μl of Cell-Tak (Corning; Cat#354240) solution at a concentration of 22.4 μg/ml and kept at 4°C overnight. Then, Cell-Tak coated Seahorse microplates wells were rinsed with distilled water and AML cells were plated at a density of 10⁵ cells per well with XF base minimal DMEM media containing 11 mM glucose, 1 mM pyruvate and 2 mM glutamine. Then 180 μl of XF base minimal DMEM medium was placed into each well and the microplate was spanned at 80 g for 5 min. After one hour incubation at 37°C in CO2-free atmosphere, basal oxygen consumption rate (OCR, as a mitochondrial respiration indicator) and extracellular acidification rate (ECAR, as a glycolysis indicator) were performed using the XFp analyzer.

**ATP analysis**
ATP was measured using the Promega Cell Titer Glo kit and protocol. Briefly, following treatment, 50,000 cells were resuspended in 80 µL and distributed in a 96 well plate. Cells were then treated in 5 replicates with vehicle/control (PBS) or sodium iodoacetate both alone or in combination with oligomycin or FCCP. Following a 1-hour incubation, 100 µL of Cell Titer Glo reaction mix solution were added to each well for a final volume of 200 µL. Plates were then analyzed by luminescence with a Perkin Elmer Luminoscan. By comparing the different conditions, total ATP and percentages of both glycolytic and mitochondrial ATP were determined.

**Determination of fatty acid oxidation**

Cells were incubated in duplicate for 3 h at 37°C with [1-14C] palmitate (0.1 mCi/ml; PerkinElmer, Boston, MA) and cold palmitate (80 µM; Sigma). Incubation buffer also contained 125 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1.25 mM KH2PO4, 1.25 mM MgSO4, 25 mM NaHCO3, 10 mM L-Carnitine and 3% fatty acid-free BSA (Sigma). After incubation, FA oxidation was measured by 14CO2 trapped in 300 µL of 1 M benzethonium hydroxide (Sigma). Radioactivity of 14CO2 was determined by liquid scintillation counting and normalized for cell number.

**NMR analysis of exometabolome**

Supernatants from culture were prepared as follows: 60 µL of a 10mM trimethylsilyl propionate (TMSP) and 2 µL of 7.6N deuterium chloride were added to 538 µL supernatant samples. Solutions were mixed thoroughly and 550 µL were then transferred to 5mm NMR tubes. NMR experiments were carried out on a Bruker 500 MHz NMR spectrometer equipped with a 5 mm TXI and a SampleXpress autosampler, enabling high throughput data acquisition for large collections of samples. The temperature was controlled at 27°C throughout the experiments. Standard 1H 1D NMR pulse sequence nuclear overhauser effect spectroscopy (NOESY) with z-gradient (Bruker pulse program noesypppr1d) was applied on each sample to obtain corresponding metabolic profiles. A total of 64 transient free induction decays (FID) were collected for each experiment with a spectral width of 14 ppm. The relaxation delay was set to 4 s. The 90° pulse length was automatically calibrated for each sample at around 10 µs. The NOESY mixing time was set to 10 ms. The total acquisition time of each sample was 9 min 53 sec.

Data processing and metabolites quantification. All FIDs were multiplied by an exponential function corresponding to a 0.3 Hz line-broadening factor, prior Fourier transformation. 1H-NMR spectra were manually phased, baselined and referenced to the TSP signal (δ = 0 ppm) using Topspin 3.5 (Bruker GmbH, Rheinstetten, Germany). Signals at 5.21 (α-glucose), 4.63 (β-glucose), 2.43 (pyruvate) and 1.41 ppm (lactate) were integrated in order to calculate the metabolite concentrations.

**Quantification of intracellular metabolites by mass spectrometry**

Extraction of metabolites was performed via fast filtration. 1 million of cells was harvested by vacuum filtration (nylon, 0.45 µm pore size, 47 mm, Sartorius, Göttingen, Germany) and washed with the same volume of NaCl (0.9%, room temperature) solution (the whole filtration procedure including the washing was completed in less than 30 s). Metabolism was quenched by placing the filter containing the cells in a centrifugation tube filled with 3 mL of a cold (-80°C) mixture of 80% methanol and 20% water. After 15 min at -80°C, the samples were centrifuged (2,000 g, -10°C, 15 min) and the supernatant was collected. The cell pellet was extracted twice more with 1 mL of the quenching solution, centrifuged 5 min and the 2 mL supernatant was added to the former one. The samples were lyophilized and dissolved in 200µL water then stored at -80°C until analysis.

Analysis of intracellular metabolites was performed as described previously (Scotland et al., 2013). Briefly, analysis was performed by high performance anion exchange chromatography (Dionex ICS 2000 system, Sunnyvale, USA) coupled to a triple quadrupole QTrap 4000 (Applied Biosystems, Foster City, USA) mass spectrometer. All samples were analyzed in the negative mode giving [M-H]- ions, which were monitored in the multiple reaction monitoring (MRM) mode. The injection volume was 15 µL. To get highly accurate quantification, the Isotope-Dilution Mass Spectrometry (IDMS) method was applied (1). For this purpose, 200 µL of IDMS standard was added directly into the quenching solution.

**OxPhos shift assay using culture in galactose**

U937 cells were cultivated in two different media for 2 weeks (equivalent to 5 consecutive passages): either in classical MEMα used in this study or in MEMα with galactose (5.6 mM) instead of glucose but with no other modification. After this adaptation, the cells were plated in 24 wells plates with 2 mL of cells at 0.3 million cells/ml and treated with PBS or AraC (0.5 µM and 1 µM) for 24h and 48h. Cell viability, proliferation and apoptosis, metabolites quantification in supernatants, ATP levels...
and OCR were assessed as described for the other experiments in this study.

**Doubling time in galactose**

U937, MOLM14, KG1a, HL60 cells were cultivated in glucose or galactose media, as described above section, for 3 days (equivalent to 2 consecutive passages) and then plated in 6 wells plates with 3 mL of cells at 0.3 million cells/mL. Cell density after 24h and 48h of treatment were quantified with trypan blue count and doubling time was assessed for each cell line using the following formula: 
\[
\text{DoublingTime} = \frac{(\text{duration} - \log_2(2))}{\log_2(\text{Final Concentration})} - \log_2(\text{Initial Concentration})
\]

**Immunophenotype analysis of AML patient specimens**

Patient samples were immunophenotyped using flow cytometry. Cells were washed in Hanks balanced salt solution with 2% FBS, stained with V500-conjugated AnnexinV, APC-conjugated anti-hCD38, Alexa Fluor 700-conjugated anti-hCD34, APC-H7-conjugated anti-hCD45 PE-Cy7-conjugated anti-hCD44, PE-conjugated anti-hCD33, V450-conjugated anti-hCD36 and ECD-conjugated anti-hCD123 (all antibodies from BD) and analyzed by Cytoflex (Beckman Coulter) with CytExpert software.

**RNA microarray and bioinformatics analyses**

**Dataset 1 (AraC residual transcriptomes):** Human CD45⁺CD33⁺ were isolated using cell sorter cytometer from engrafted BM mice (for 3 primary AML specimens) treated with PBS or treated with AraC. RNA from AML cells was extracted using Trizol (Invitrogen) or RNeasy (Qiagen). RNA was amplified before array analysis (NuGEN Technologies). The Affymetrix HuGene_1.0_st chip was used to assess mRNA expression. Data were normalized by robust multichip average (RMA – [34]) using GeneSpring GX (Agilent) and imported into R statistical environment (http://www.R-project.org/) for further analysis. First, the quality of the data was assessed using standard methods (2). Second, principal variance component analysis (PVCA) (3) was applied to estimate the percentage of variability related to treatment and batch. As PVCA revealed a strong batch effect, data was submitted to ComBat (4) for batch effect correction. Finally, the Rank Product algorithm (5) was used to identify gene affected by AraC. Genes with pfp < 0.05 and absolute fold change >= 1.5 were considered as differentially expressed.

**Dataset 2 (High OXPHOS transcriptomes):** Total RNAs were extracted from 4 AML cell lines (MOLM14, HL60, U937, KG1a) untreated and treated with 10 mM metformin 24hr in independent triplicata using the Qiagen kit according to the manufacturer’s instructions. RNA purity and integrity were monitored using NanoDrop® ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer with RNA 6000 Nano assay kit. Only RNAs with no sign of contamination or marked degradation (RIN > 9) were considered good quality and used for further analysis. Transcriptome profiling assays were performed using the Affymetrix HuGene_2.0_st arrays. Briefly, 250 ng of total RNAs were reverse transcribed into cDNA, then transcribed into cRNAs and labeled into biotinylated cRNA using the GeneChip WT PLUS Reagent kit (Affymetrix) according to the manufacturer’s standard protocols (P/N 4425209 Rev.B 05/2009 and P/N 702808 Rev.6). Labeled cRNA products were randomly fragmented and hybridized onto Affymetrix GeneChips. Upon hybridization, arrays were washed and stained using the Affymetrix GeneChip WT Terminal Labeling and Hybridization kit, before being scanned using a GeneChip Scanner 3000. CEL files were first imported into Partek Genomics SuiteTM (GS) 6.6 for preprocessing using default settings. After preprocessing, data were transferred into R environment for quality control, analyze of variance and differential expression analysis. Quality control and analyze of variance were done as previously and did not show any anomaly. Therefore, LIMMA package (6) with standard procedure (7) was used for differential expression analysis. Resulting p-values were adjusted for multiple testing error with Benjamin and Hochberg FDR (8). Genes with FDR < 0.05 and absolute fold change >= 1.5 were considered of interest.

Clustering and heat maps were generated using Ingenuity Pathway Analysis and Genomics software’s. Lists of DE mRNA (FC>+/−1.5 and FDR<0.05) obtained throughout the study were uploaded in the Genome Analyzer bioinformatics tool (Genomatix, www.genomatix.de) for further functional analyses (GO term, transcription factors and small molecules) based on the Genomatix literature mining. The significance of the association between each list and functions or canonical pathways was measured by Fisher’s exact test. As a result, a p-value was obtained, determining the probability that the association between the genes in our data set and a function or canonical pathway can be explained by chance alone. Transcriptional gene regulatory networks were built based on the molecular relationship repertoire referenced in the Genomatix library. The Genomatix Upstream Regulators analytic was also used to identify the transcriptional regulators that could explain the experimental gene expression patterns, predict their activation state, and determine the biological
functions affected by the regulatory cascade. Complementary functional analyses were performed with Davis and FUNRICH.

Gene set enrichment analysis was performed using GSEA v2.0 tool developed by the Broad Institute (9). The enrichment scores (ES) were computed for the ranked genes from the AraC screen or High OXPHOS/metformin screen.

**Dataset 3 (Low responder transcriptomes):** Total RNAs were extracted from 21 AML patient samples using the NucleoSpin® RNA kit (Macherey-Nagel) according to the manufacturer’s instructions. RNA purity and integrity were monitored using NanoDrop® ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer with RNA 6000 Nano assay kit. Only RNAs with no sign of contamination or marked degradation (RIN > 9) were considered good quality and used for further analysis. Transcriptome profiling assays were performed using the Affymetrix HuGene_2.0_st arrays. Briefly, 250 ng of total RNAs were reverse transcribed into cDNA, then transcribed into cRNAs and labeled into biotinylated cRNA using the GeneChip WT PLUS Reagent kit (Affymetrix) according to the manufacturer’s standard protocols (P/N 4425209 Rev.B 05/2009 and P/N 702808 Rev.6). Labeled cRNA products were randomly fragmented and hybridized onto Affymetrix GeneChips. Upon hybridization, arrays were washed and stained using the Affymetrix GeneChip WT Terminal Labeling and Hybridization kit, before being scanned using a GeneChip Scanner 3000. Preprocessing and quality control were made as in Dataset 2 and RankProduct was used for differential expression analysis. Genes with PIP < 0.05 and absolute fold change >= 1.5 were considered of interest in Low or High responding patients (LowR versus HighR, respectively) assessed according to AraC response in our PDX model.

Transcriptomic data have been deposited at the Gene Expression Omnibus (GEO) database under accession number GSE97631 (PDX post-AraC, dataset 1), GSE97346 (AML cell lines, dataset 2), and GSE97393 (primary AML patients, dataset 3).

**Targeted re-sequencing**

A panel of 40 genes frequently mutated in AML and myeloid malignancies was designed. Amplicon libraries were obtained from 112 ng DNA per sample, using HaloPlex Target Enrichment System® (Agilent technologies), according to the manufacturer’s protocol except that all volumes of reactions were halved. Sequencing was performed using a MiSeq® sequencer (Illumina) using manufacturer recommendations. Results were analyzed after alignment of the readings using the SureCall® Software version 3.0.1.4 from Agilent Technologies. BWA MEM algorithm was used for alignment and IlluminaNC SNP PET SNP Caller algorithm was used to identify SNP and INDELS variants. Minimum allele frequency for variant calling was set at 2% with a minimum local depth at 40. All variants from diagnosis samples were manually checked in all samples using IGV version 2.3 software for low frequency variants monitoring. Complementary detection of described polymorphisms was performed by an in-house software using Ensembl database.

**All publicly accessible transcriptomic databases of AML patients used in this study:**


**Supplementary References**


**Supplementary Table Legends**

**Supplementary Table S1.** (1) Clinical characteristics of 27 AML patients used in this study for engraftment in NSG mice, including the 25 ones treated in vivo with 60 mg/kg/d cytarabine (AraC) for 5 days. (2) Clinical and mutational characteristics of AML cell lines used in this study.

**Supplementary Table S2.** Determination of LICs frequency in bone marrow and spleen from AraC- or PBS-treated AML-xenografted NSG mice using in vivo limiting dilution assay.

**Supplementary Table S3.** (1) Gene signatures of AraC residual AML blasts in vivo from three AML patients xenografted and treated with AraC. (2) Functional analyses of up-regulated genes of AraC residual AML blasts in vivo. (3) Functional analyses of down-regulated genes of AraC residual AML blasts in vivo. (4) Prognostic analysis of the up- and down-regulated gene signatures of AraC-residual AML blasts in vivo in three independently published cohorts of AML patients.

**Supplementary Table S4.** (1) HIGH OXPHOS gene signature generated from HIGH versus LOW OXPHOS AML cell lines untreated or treated with metformin. (2) Functional analysis of upregulated genes encoding for mitochondrial proteins in untreated HIGH versus LOW OXPHOS AML cell lines.

**Supplementary Table S5.** Up- and down-regulated genes in AML patients that have the lower response (LowR) to AraC in vivo in NSG mice compared to high responder (HighR) to AraC in vivo using PDX assay.

**Supplementary Table S6.** HIGH CD36 gene signature generated from upregulated genes in AML patients that express the most CD36 mRNA compared to that of the lowest expression of CD36 in TCGA cohort.
Supplementary Table S7. Summary of all GSEA data identified in AraC- versus PBS-treated AML cells in PDX and LOW versus HIGH Responder to AraC in PDX.

Supplementary Figure Legends

Supplementary Figure S1. Clinical distributions of AML patients from TUH and 3 independent patient cohorts. A-B, AML patients from database cohorts were classified according to their clinical data: FAB classification (A) and cytogenetics risk groups (B). C, AML patients studied in our NSG-based PDX models were classified according to their mutational status of FLT3, NPM1, and DNMT3A. D, percentage of human AML engraftment in NSG mice was calculated in bone marrow and in spleen separately for each primary AML patient samples tested in this study.

Supplementary Figure S2. In vivo treatment with 60 mg/kg/d of cytarabine (AraC) given daily for 5 days induces a significant reduction of the total cell tumor burden in AML-engrafted mice. A, Kaplan-Meyer curves of overall survival in NSG mice treated with AraC escalating doses (60, 90 and 120 mg/kg/d) via IP injection for 5 days. B-C, Total number of human AML cells expressing CD45 and CD33 in NSG mice treated 5 days with AraC escalating doses compared to vehicle (PBS)-treated xenografted mice was analyzed and quantified using flow cytometry in peripheral blood (blast/µl; B) weekly for 4 weeks after the last AraC administration and in bone marrow and spleen (total cell tumor burden in Millions; C) at day 8. D, Total number of human AML cells expressing CD45 and CD33 in bone marrow and spleen (total cell tumor burden in Millions) was analyzed and quantified using flow cytometry in AraC-treated xenografted mice with 60 mg/kg/d given daily via IP injection for 3, 5 and 7 days compared to vehicle (PBS)-treated xenografted mice. E, Complete blood count (CBC) of NSG mice treated with 60, 90 and 120 mg/kg/d during 5 days, including white blood cell count (WBC), hemoglobin (Hbg) and platelet count (PLT), was analyzed using an automated analyzer. P values were determined by the Mann-Whitney test. n.s., not significant. p-value: *≤0.05, **≤0.01, ***≤0.001.

Supplementary Figure S3. In vivo cytarabine (AraC) treatment induces significant but heterogeneous response in bone marrow and spleen of AML-xenografted NSG mice. A-B, Flow cytometry analysis of bone marrow and spleen for total viable human CD33+CD45+ cells in AML-engrafted mice for each primary AML patient samples separately (A) and for all patients (B). Fold reduction of tumor engraftment in AraC-treated mice compared with control-treated mice was calculated in bone marrow and spleen, separately. C, Goldner staining of bone marrow (sternum section) at 2.5x or 20x shows humal AML engraftment in PBS/vehicle-treated AML xenografted mice and tumor burden reduction in matched AraC-treated AML-xenografted mice. D, Hematoxylin and eosin staining of bone marrow (femur section) at 40x shows high AML engraftment level in vehicle (PBS)-treated mice and tumor burden reduction in AraC-treated mice. P values were determined by the Mann-Whitney test. n.s., not significant. p-value: *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001.

Supplementary Figure S4. Comparative analysis of the in vivo response to cytarabine (AraC) with clinico-biological data of AML patients. For each primary AML patient samples, fold reduction of tumor engraftment in AraC-treated mice compared with vehicle-treated mice was calculated. In vivo response to AraC of AML patients were analyzed according to their clinical data: A, at diagnosis or relapse, B, the patient gender, C, the patient age at diagnosis, D, patients who reached or not complete remission (CR) after chemotherapy, E, the FAB classification, F, the expression of CD34, G, the cytogenetics risk group, H, the mutational status of FLT3, I, the FLT3 allelic burden, J, the mutational status of NPM1, DNMT3A and IDH1. K, correlation between in vitro response to AraC assessed by in vitro dose response (EC50 24h or 48h) of each AML patient samples and their respective in vivo response to AraC assessed either by tumor burden reduction or apoptosis induction in NSG mice. p-value: *≤0.05.

Supplementary Figure S5. In vivo cytarabine (AraC) treatment induces changes in CD34+/CD38− phenotypes in AML-engrafted mice. A, Gating strategy using untreated mouse bone human cells are gated by forward and side scatter. Doublets are excluded. Human cells are gated on by CD45 and high side scatter. Fluorescent controls were used to define CD38 negative and CD34 negative gates. B, Representative flow cytometry plots of human CD34 CD38 populations in bone marrow from mice engrafted with human AML sample.
(Ps1) after AraC or vehicle treatment. Flow cytometric analyses of human viable (AnnexinV/’7-AAD’) CD45^CD33^ residual AML cells were performed to assess the expression level of CD34 and CD38 in CD45^CD33^ (C-D) and CD34^ (E-F) cell populations in bone marrow from AraC-treated xenografted mice compared to vehicle (PBS)-treated xenografted mice. P values were determined by the Mann-Whitney test. n.s., not significant. n.d., not determined. p-value: *≤0.05, **≤0.01.

Supplementary Figure S6. In vivo cytarabine (AraC) chemotherapy treatment leads to reduction of the absolute number of human CD45^CD38^+ populations in AML. 
A-B, Total number of human AML cells expressing CD34^CD38^ and C-D, CD34^CD38^ in residual human CD45^CD33^ cell lines analyzed and quantified using flow cytometry in bone marrow and spleen from AraC-treated xenografted mice compared to vehicle (PBS)-treated xenografted mice. P values were determined by the Mann-Whitney test. n.s., not significant. n.d., not determined. p-value: *≤0.05, **≤0.01, ***≤0.001

Supplementary figure S7. No enrichment in G0 quiescent cells was observed in mice treated with sublethal dose of cytarabine (AraC) for 5 days in vivo.
A, Gating strategy using untreated mouse bone marrow. Dead cells are excluded with 7AAD staining. Human cells are gated by forward and side scatter. Doublets are excluded. Monocytes are gated on by CD45 and high side scatter. G0 is defined by low Pyronin Y and Hoechst Staining. B-C, Percentage of human AML G0 cells in residual human CD45^CD33^CD45^CD38^ was analyzed and quantified using flow cytometry in bone marrow from AraC-treated xenografted mice compared to vehicle (PBS)-treated xenografted mice. D, Total number of human AML G0 cells in residual human CD45^CD33^ population analyzed and quantified using flow cytometry in bone marrow from AraC-treated xenografted mice compared to vehicle (PBS)-treated xenografted mice. E, Hoechst/Pyronin Y-based flow cytometric assay were performed to determine the percent of cells in G0 from residual human CD45^CD33^ AML cells in vivo after AraC treatment with 1000 mg/kg in AML xenografted mice compared to vehicle (PBS)-treated AML xenografted mice. Flow cytometric analyses of human CD45^CD33^ residual AML cells were performed to assess: F, total number of human AML cells in peripheral blood (blasts/µl) and in G, bone marrow and spleen (Millions). p-value: *≤0.05. H, Schematic representation of the limiting dilution analysis used in PDXs to assess the frequency of LICs after AraC or PBS treatment in vivo. AML blasts engrafted in first recipient NSG mice and treated or not with AraC 60mg/kg/d for 5 days were injected in a second recipient to assess the LICs frequency.

Supplementary figure S8. Mitochondrial and energetic features of LOW (KG1, KG1a, U937) and HIGH (MOLM14, MV4-11, HL60) OXPHOS AML cell lines.
Six cells lines were classified according to their oxidative phosphorylation and energetic status as measured by their A, mitochondrial respiration (basal oxygen consumption rate, OCR assessed by Clark electrode measurement) and mitochondrial ATP production, B, mitochondrial ROS content and mass assessed by flow cytometry (MitoSOX and TMRE fluorescent probes) and C, Mitochondrial ROS and membrane potential determined by flow cytometry (MitoSOX and TMRE fluorescent probes). D, Doubling time of four AML cell lines grown in minimal MEMa medium supplemented with glucose or galactose.

Supplementary figure S9. Functional analysis of the transcriptomes of LOW (KG1a, U937) versus HIGH (MOLM14, HL60) OXPHOS AML cell lines untreated or treated with metformin. 
A, Four cell lines were classified according to their oxidative phosphorylation and energetic status by measuring mitochondrial respiration (basal oxygen consumption rate, OCR assessed with a Clark electrode) and mitochondrial ATP production (% of intracellular total ATP) after mitochondrial respiratory chain complex 1 inhibition (10 mM Metformin for 24h treatment), inducing energetic shift (eg. Pasteur effect) and leading to LOW OXPHOS profile for HIGH OXPHOS AML cell lines. B, Comparative transcriptomic analysis on HIGH (HL60, MOLM14) versus LOW (U937, KG1a) OXPHOS AML cell lines in basal condition. C, Gene ontology (GO) classification in biological pathways of up-regulated signatures in untreated HIGH OXPHOS compared to untreated LOW OXPHOS cell lines using FunRich software. D, GO classification in 177 genes encoding for mitochondrial proteins of up-regulated signatures in HIGH OXPHOS compared to LOW OXPHOS cell lines using FunRich software. E, HIGH OXPHOS gene signature (221 transcripts) was identified through a transcriptomic analysis of untreated HIGH versus untreated LOW OXPHOS cells (1702 genes) and untreated HIGH versus metformin-treated HIGH OXPHOS cells (381 genes). F, GO classification and biological processes of this HIGH OXPHOS gene signature were identified by Genomics software analysis.
Supplementary figure S10. AML cells surviving after cytarabine (AraC) treatment are resistant to chemotherapies and are pre-existing CD36"CD44" phenotype with an increased oxidative metabolism. A-B, In vivo AraC-treated residual AML cells were sorted and treated with AraC, idarubicin or VP16 for 48h to establish EC_{50} compared to their matched PBS-treated AML cells. C, Schematic representation of the chemotherapy treatment used to sort high ROS (DCF) and low ROS (DCF) cells. AML cell lines or primary AML samples were either treated in vitro at 2 µM AraC for 24h or injected into mice. Mice were then treated with PBS or AraC (30mg/kg/d for CLDXs and 60mg/kg/d for PDXs) for 5 days and were sacrificed at day 8. Cells were FACs-sorted based on intracellular ROS levels using DCF-DA probe and ex vivo AraC sensitivity was then evaluated. D, EC_{50} of the sorted ROS fractions (L: low DCF sorted fraction, H: high DCF sorted fraction) for AraC. Flow cytometric analyses of human CD45"CD33" residual AML cells in AraC-treated AML-xenografted mice at Day 3-5-8 compared to PBS-treated xenografted mice were performed to assess apoptosis induction using AnnexinV/7AAD staining (E), the total number of human AML cells in bone marrow and spleen (Millions; F), total ROS content using DCF-DA probe (G), mitochondrial mass using MTG probe (H), mitochondrial membrane potential using TMRE probe (I), the expression level of CD36 (J), CD44 (K) and CD123 (L). M-N, Variant allele frequency (VAF) of different mutations was analyzed in AML cells of Ps22 and Ps19 from diagnosis, relapse and PDX model at different time points during AraC treatment using targeted NGS sequencing. P values were determined by the Mann-Whitney test. n.s., not significant, *≤0.05, **≤0.01, ***≤0.001.

Supplementary figure S11. Culture in galactose induces energetic shift of LOW OXPHOS AML U937 cells toward HIGH OXPHOS state, leading to cytarabine (AraC) resistance in AML. A, Schematic diagram of galactose treatment inducing energetic shift of LOW OXPHOS cell line (U937) toward HIGH OXPHOS state. B, Oxygen consumption rate was assessed using Clark electrode after 24h of treatment either with glucose or galactose medium and either with PBS, 0.5 µM or 1 µM of AraC. C, Metabolites (pyruvate, glucose and lactate) were quantified by NMR spectrometry on supernatants of treated cells. D-E, Percent of mitochondrial and total ATP production was measured using cell titer glo kit after 24h of treatment. F-G, Cell density and cell viability after 24h or 48h of treatment were quantified with trypan blue count. H, Apoptosis induction was measured using AnnexinV/7AAD staining by flow cytometry after 24h or 48h of treatment. P values were determined by the Mann-Whitney test. n.s., not significant. p-value: *≤0.05, **≤0.01, ***≤0.001.

Supplementary figure S12. Energetic shift of mtDNA-depleted Rho p0 MOLM14 cells toward LOW OXPHOS state induces AraC sensitivity. A, MOLM14 cells (M14) are treated with ethidium bromide to generate mtDNA-depleted Rho zero cells (M14p0). B, Expression of mitochondrial encoding proteins (PGC1α, ND6 and complex IV) was evaluated by western blot. C-E, Basal oxygen consumption rate was analyzed using Clark electrode as well as proton leak (D) and spare respiratory capacity (SRC, E) in response to ETC inhibitors: oligomycin (2 µM), CCCP (5 µM), Rotenone and Antimycin A (1 µM). F, ATP production turnover was measured using cell titer Glo viability kit. G, AraC sensitivity at 24h (EC_{50}) was determined by AnnexinV/7-AAD staining using flow cytometry.

Supplementary figure S13. Electron Transfer Chain Complex I inhibition by Phenformin (Phenf) induces energetic shift toward LOW OXPHOS state and increases cytarabine (AraC) sensitivity in MOLM14 cells. A, Schematic diagram of the decrease of OXPHOS capacity by Phenf treatment that leads to an energetic shift toward LOW OXPHOS state and chemosensitizes MOLM14 cells to AraC. B, Basal oxygen consumption rate was assessed using Seahorse XFp after 24h of treatment with Phenf (750 µM) and AraC (2 µM). C, Metabolites (pyruvate, glucose and lactate) were quantified by NMR on supernatants of treated cells. D, Percent of glycolytic ATP production was measured using cell titer glo viability kit after 24h of treatment. E, Protein expression of oxidative phosphorylation (OXPHOS) complexes was assessed by western blot after 24h treatment. F, Mitochondrial ROS were measured by flow cytometry using mean of fluorescence (MFI) of MitoSOX fluorescent probe in viable cells. G-H, Cell density and cell viability after 24h of treatment was quantified with trypan blue count. I, Apoptosis induction was measured using AnnexinV/7AAD staining in flow cytometry. J, Loss of MMP was measured using TMRE staining in flow cytometry. K, Anti- and pro-apoptotic proteins levels were quantified by western blot method. P values were determined by the Mann-Whitney test. n.s. not significant. n.d. not determined.*P≤0.05; **P≤0.01.
**Supplementary figure S14.** Electron Transfer Chain Complex I inhibition by Metformin (Met) induces energetic shift toward LOW OXPHOS state and increases cytarabine (AraC) sensitivity in MOLM14 cells.  
A, Schematic diagram of the decrease of OXPHOS capacities by Met treatment that leads to an energetic shift toward LOW OXPHOS state and chemosensitizes MOLM14 cells to AraC.  
B, Basal oxygen consumption rate was assessed using Clark electrode after 24h of treatment with Met (10 mM) and AraC (2 µM).  
C, Metabolites (pyruvate, glucose and lactate) were quantified by NMR on supernatants of treated cells.  
D-E, Mitochondrial membrane potential (MMP) and mitochondrial ROS were measured by flow cytometry using mean of fluorescence (MFI) of TMRE or MitoSOX fluorescent probes in viable cells, respectively.  
F-G, Cell density and cell viability after 24h of treatment were quantified with trypan blue count.  
H, Apoptosis induction was measured using AnnexinV/7AAD staining in flow cytometry.  
I, Loss of MMP was measured using TMRE staining in flow cytometry.  
P values were determined by the Mann-Whitney test. n.s. not significant. n.d. not determined. *P≤0.05; **P≤0.01.

**Supplementary figure S15.** Electron Transfer Chain Complex I inhibition by Rotenone (Rot) induces energetic shift toward LOW OXPHOS state and increases cytarabine (AraC) sensitivity in MOLM14 cells.  
A, Schematic diagram of the decrease of OXPHOS capacities by Rot treatment that leads to an energetic shift toward LOW OXPHOS state and chemosensitizes MOLM14 cells to AraC.  
B, Basal oxygen consumption rate was assessed using Clark electrode after 24h of treatment with Rot (200 µM) and AraC (2 µM).  
C, Metabolites (pyruvate, glucose and lactate) were quantified by NMR on supernatants of treated cells.  
D-E, Mitochondrial membrane potential (MMP) and mitochondrial ROS were measured by flow cytometry using mean of fluorescence intensity (MFI) of TMRE or MitoSOX fluorescent probes in viable cells.  
F-G, Cell density and cell viability after 24h of treatment were quantified with trypan blue count.  
H, Apoptosis induction was measured using AnnexinV/7AAD staining in flow cytometry.  
I, Loss of MMP was measured using TMRE staining in flow cytometry.  
P values were determined by the Mann-Whitney test. n.s. not significant. n.d. not determined. *P≤0.05; **P≤0.01.

**Supplementary figure S16.** Electron Transfer Chain Complex III inhibition by Antimycin A (AntiA) induces energetic shift toward LOW OXPHOS state and increases cytarabine (AraC) sensitivity in MOLM14 cells.  
A, Schematic diagram of the decrease of OXPHOS capacity by AntiA treatment that leads to an energetic shift toward LOW OXPHOS state and chemosensitizes MOLM14 cells to AraC.  
B, Basal oxygen consumption rate was assessed using Clark electrode after 24h of treatment with AntiA (10 µM) and AraC (2 µM).  
C, Metabolites (pyruvate, glucose and lactate) were quantified by NMR on supernatants of treated cells.  
D-E, Mitochondrial membrane potential (MMP) and mitochondrial ROS were measured by flow cytometry using mean of fluorescence intensity (MFI) of TMRE or MitoSOX fluorescent probes in viable cells, respectively.  
F-G, Cell density and cell viability after 24h of treatment are quantified with trypan blue count.  
H, Apoptosis induction was measured using AnnexinV/7AAD staining in flow cytometry.  
I, Loss of MMP was measured using TMRE staining in flow cytometry.  
P values were determined by the Mann-Whitney test. n.s. not significant. n.d. not determined. *P≤0.05; **P≤0.01.

**Supplementary figure S17.** Electron Transfer Chain Complex III inhibition by Atovaquone (ATQ) induces energetic shift toward LOW OXPHOS state and increases cytarabine (AraC) sensitivity in MOLM14 cells.  
A, Schematic diagram of the decrease of OXPHOS capacity by ATQ treatment that leads to an energetic shift toward LOW OXPHOS state and chemosensitizes MOLM14 cells to AraC.  
B, Basal oxygen consumption rate was assessed using Clark electrode after 24h of treatment with ATQ (20 µM) and AraC (2 µM).  
C, Metabolites (pyruvate, glucose and lactate) were quantified by NMR on supernatants of treated cells.  
D-E, Mitochondrial membrane potential (MMP) and mitochondrial ROS were measured by flow cytometry using mean of fluorescence intensity (MFI) of TMRE or MitoSOX fluorescent probes in viable cells, respectively.  
F-G, Cell density and cell viability after 24h of treatment are quantified with trypan blue count.  
H, Apoptosis induction was measured using AnnexinV/7AAD staining in flow cytometry.  
I, Loss of MMP was measured using TMRE staining in flow cytometry.  
P values were determined by the Mann-Whitney test. n.s., not significant. n.d., not determined. *P≤0.05; **P≤0.01.
Supplementary figure S18. Working model of the resistance to AraC in vivo.

A, Blood Spot and Jung et al. (2015) transcriptomic databases were used to compare CD36 mRNA expression in human AML cells to normal hematopoietic stem cells (HSC) from healthy donor. In Jung Cohort, Progenitor Cells (LPC) and Stem Cells (LSC) were segregated from leukemic blast according to their immunophenotype status. B, Kaplan-Meier survival curve of AML patients from TCGA cohort that express CD36 mRNA at the highest level (red) or the lowest level (green). Log-Rank p-values are displayed. C, OXPHOS and FAO related gene set enrichment (GSEA) in TCGA cohort. Patients from this cohort were sorted in function of their CD36 mRNA expression. D, High CD36 gene set has been analyzed for this enrichment in specific biological pathways and sub-pathways with Genomatix software. E, Kaplan-Meier survival curve of AML patients that overexpress FAO related gene sets expression (highest in red) in TCGA patient cohort. Log-Rank p-values are displayed. F, Schematic diagram of mechanisms-of-resistance to AraC of AML cells in the in vivo context of the hematopoietic niche where bone marrow adipocytes and MSCs support and maintain HIGH OXPHOS energetic metabolism of AML cells and their survival upon AraC. P values were determined by the Mann-Whitney test. n.s., not significant. n.d., not determined. p-value: *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001.
Figure S1
**Figure S2**

**A**

Mice survival (%)

- Vehicle (n=6)
- 60mg/kg x 5 (n=5)
- 90mg/kg x 5 (n=6)
- 120mg/kg x 5 (n=6)

**B**

hCD33*CD45* cells in PB

- AraC (mg/kg)

**C**

hCD33*CD45* cells in PB

- PBS
- 10
- 30
- 60

**D**

hCD33*CD45* cells in BM+SP

- D3
- D8
- D8

**E**

- WBC
- Hgb
- PLT

- Weeks after the first dose
- Weeks (post-Tx)
In vivo response to AraC
Tumor burden (Fold change)

Dx/Rel
- n=20
- n=5

Gender
- M
  - n=12
- F
  - n=13

Age at Dx
- <64
  - n=11
- ≥64
  - n=9

Post 3+7
- Failure
  - n=1
- CR
  - n=13

FAB classification
- M1
  - n=9
- M2
  - n=3
- M4/M5
  - n=9
- NOS/MLD
  - n=4

CD34
- Neg
  - n=6
- Dim
  - n=6
- Pos
  - n=12

Cytogenetics risk group
- Favorable
  - n=2
- Intermediate
  - n=9
- Unfavorable
  - n=2

FLT3 status
- WT
  - n=7
- Mut
  - n=13

FLT3 allelic burden
- <50%
  - n=5
- ≥50%
  - n=4

Mutational status
- NPM1
  - WT
  - n=2
  - Mut
  - n=5
  - Mut (ITD) others
  - n=4
- DNMT3A
  - WT
  - n=8
  - Mut
  - n=2
- IDH1
  - WT
  - n=8
  - R132
  - n=2

In vitro response to AraC
- 24h
  - EC50 (µM)
  - r=-0.1981
  - p=0.40
- EC50 (µM)
  - r=-0.262
  - p=0.30
- EC50 (µM)
  - r=0.03974
  - p=0.8940

In vivo response to AraC
- Tumor burden (Fold change)
- Apoptosis (Fold induction)
- Tumor burden (Fold change)

Figure S4
Figure S6
Figure S8

- **A**: Mitochondrial OCR (nmol O₂/min/10⁶ cells) for LOW and HIGH OXPHOS.
- **B**: Mitochondrial ROS (mitoSOX, relative intensity) for LOW and HIGH OXPHOS.
- **C**: Mitochondrial membrane potential (TMRE, relative intensity) for LOW and HIGH OXPHOS.
- **D**: Doubling time (hours) for glucose and galactose in M4, HL60, U937, KG1a.

Legend:
- MOLM14
- HL60
- KG1a
- U937
Figure S9

**A**

+ metformin (10mM, 24hr)

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<tr>
<th>KG1a</th>
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<th>MOLM14</th>
<th>HL60</th>
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**B**

mRNA expression (Log2FC High/Low)

-5 -4 -3 -2 -1 0 1 2 3 4 5 6

**C**

Up-regulated Biological Pathways – 1923 genes

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<th>Pathway</th>
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**D**

Up-regulated Mitochondrial Functions – 177 genes

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<td>Eukaryotic Translation Elongation</td>
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<td>Fatty Acyl-CoA Biosynthesis</td>
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<td>Mitochondrial transcription initiation</td>
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<td>Ubiquinone-10 biosynthesis (eukaryotic)</td>
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<td>Activation and oligomerization of BAK protein</td>
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**E**

HIGH OXPHOS
LOW OXPHOS
HIGH OXPHOS
HIGH OXPHOS + Metformin

1702 221 381

HIGH OXPHOS Gene signature

**F**

HIGH OXPHOS signature – 221 genes

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Figure S9
Figure S15
Figure S16
Atravaquone

Oxygen

Pyruvate

Glucose

Lactate

Active MMP

Mitochondrial $O_2^-$

Cell density

Cell viability

Apoptosis

Loss of MMP

Figure S17
Figure S18

**C**

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**E**

- **Fatty Acid Beta Oxidation**
  - GO - 11 genes
  - HR = 4.83
  - p-value = 0.013

- **Fatty Acid Metabolic Process**
  - GO - 63 genes
  - HR = 6.86
  - p-value = 0.016

- **Fatty Acid Triacylglycerol and Ketone Body Metabolism**
  - Reactome - 168 genes
  - HR = 20.93
  - p-value = 0.005

**F**

- Adipocyte
- Fatty Acids
- AML Cells
- CD36
- Mitochondria
- ROS
- ATP
- Chemoresistance
- Low responder to chemotherapy
- Residual disease

Figure S18