# The small-molecule compound AC-73 targeting CD147 inhibits leukemic cell proliferation, induces autophagy and increases the chemotherapeutic sensitivity of acute myeloid leukemia cells

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## Supplementary materials

### Supplementary methods

**CB CD34$^+$ HPCs purification and unilineage monocytic (Mo) and granulocytic (G) liquid cultures.** CD34$^+$ HPCs were cultured in BIT 9500 serum-free medium (Stemcell Technologies Inc. Vancouver, BC, Canada) supplemented with (i) human low density lipoprotein (40µg/ml), FLT3 ligand (100 ng/ml), IL6 (10 ng/ml) and M-CSF (50 ng/ml) for Mo cultures (ref) ; (ii) IL-3 (1 U/mL), GM-CSF (0.1 ng/mL), and G-CSF (500 U/mL) for G cultures, as previously described.[28,29] For morphologic analysis, HPCs were smeared on glass slides by cytospin centrifugation, stained with May-Grünwald-Giemsa and analyzed at 400X magnification under a microscope (Eclipse 1000, Nikon, Tokyo, Japan) equipped with a digital camera.

**Human AML cell lines culture and differentiation.** All leukemic cell lines were cultured in RPMI-1640 medium supplemented with 10% FCS, 100 µg/ml streptomycin, 100 U/ml penicillin, in a humidified atmosphere containing 5% $CO_2$ at 37°C. Monocytic-induced differentiation of U937 cells was performed by using 50 ng/ml 1α250H-Vitamin D3 (vit.D3) (Roche, Basel, Switzerland). Granulocytic-induced differentiation of HL-60 and NB4 cells was performed by using 1µM All-Trans Retinoic Acid (ATRA) (Sigma), for the indicated time points.

**CHO cell line.** CHO cell line from Chinese Hamster Ovary, used as negative control for CD147 expression, was grown in medium Ham′s F12 + 2mM Glutamine + 10% FBS (Sigma).

**Cell growth, cell cycle profile, viability and apoptosis analysis.** Cell viability, proliferation, apoptosis and cell cycle profiles were investigated in HPCs and leukemic cells, treated and control. Cells were seeded in 96-well culture plates at a density of $2 \times 10^4$ viable

cells/100 µl/well, in triplicates. Cell growth was analyzed by cell counting using trypan blue the rate of proliferation and the percentage of viable cells. Cell Titer-Glo Luminescent Assay (CTG assay, Promega, Madison, WI) was also used as a cell viability assay, to determine cell growth and viability, according manufacturer's procedures. Apoptosis was analyzed by using Annexin V-FITC and Propidium Iodide apoptosis kit, according manufacturer's instruction (BD Pharmingen, San Diego, CA), to detect both early and late apoptosis. Cell vitality and cell cycle analysis were evaluated by using assays according manufacturer's procedures (7-aminoactinomycin D and Cycletest Plus DNA detection kits, are from BD Pharmingen).

**Flow cytometry analysis.** Expression of CD14 and CD15 cell surface antigens, stained with PE-conjugated anti-mouse CD14 and CD15 antibodies (BD Pharmingen, San Diego, CA), was analyzed by flow cytometry using a FACScan Flow cytometer (Becton Dickinson, Bedford, MA) to control respectively monocytic and granulocytic differentiation, as previously described.[28,29] PE-conjugated anti-human -CD34, -CD38 and -CD371 monoclonal antibodies (BD Biosciences) and FITC-anti-CD147 antibody (Ab monoclonal, FITC-Mouse-Anti human-CD147, BD Pharmingen) were used for cell labeling and analysis of CD34, CD38, CD371 and CD147 cell surface expression. The mean fluorescence intensity (MFI) was reported as the ratio between the geometric mean fluorescence values observed in CD147 labeled cells and cells labeled with an appropriate negative control, gated on FSC/ SSC dot plot to select viable cells. The percentage of positive cells for CD34, CD38 and CD371 was calculated subtracting the values of isotype controls. FACSCAN flow cytometer with Cell Quest software (BD) was used for acquisition and analysis. The results were expressed in terms of the percentage of positive cells and of the mean fluorescence intensity (MFI).

**Quantitative real-time RT-PCR.** Total RNAs were extracted using TRIzol reagent and reverse transcribed, as described.[29] Quantitative real-time RT-PCR analysis (qRT-PCR) was

performed and normalized with the internal control β-actin (ACTB). We used commercial ready-to-use primers/probe mixes for CD147 (Basigin/ BSG) (Hs 00936295) and ACTB (Hs 9999903_m1; 20X, 4310881E) (Assays on Demand Products, Applied Biosystems), according to the manufacturer's procedures, TaqMan technology and the ABI PRISM 7700 DNA Sequence Detection System (Applied Biosystems, Foster City, CA, USA).[29]

**Western blot analysis.** Aliquots of 25 µg of total protein extract were prepared and resolved on 10% mini-Protean TGX precast gels (Bio-Rad, CA, USA) for standard denaturing electrophoresis, according manufacturer's instructions. Precast gels were then transferred to nitrocellulose filters by using Transblot-Turbo transfer system, according manufacturer's procedures (Bio-Rad, Hercules, CA, USA). Membranes were saturated for 1 h in 5% milk with 0.2% Tween 20-TBS-T (10 mM Tris–HCl pH 8.0, 150 mM NaCl) solution before incubation with a primary antibody in 1% BSA/TBS-T solution overnight at 4°C. After several washes in TBS-T buffer, blots were incubated with the corresponding peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) in TBS-T for 1 h. Bound antibodies were visualized by using the enhanced chemiluminescence technique (ECL) according to the manufacturer's instructions (Super Signal West Pico, Pierce, Rockford, IL, USA). Antibodies used were: CD147 monoclonal antibody (EMMPRIN (HM6) sc-53693, Santa Cruz, CA); for total STAT3 protein detection, STAT3 (H-190) polyclonal antibody (sc-7179, Santa Cruz); phospho-STAT3-S727 and phospho-ERK1/2 (p44/p42 MAPK) polyclonal antibodies (Immunological Sciences, RM, I); for total ERK1/2 proteins detection, p44/42 MAPK (Erk1/2) polyclonal antibody (9102, Cell Signaling Technology, Danvers, MA); for LC3-I and LC3-II proteins detection, LC3B polyclonal antibody (NB600-1384, Novus Biologicals, Novus, I). Monoclonal antibody anti-actin (Sigma-Aldrich, Milan, I) was used as an internal control of the loaded amounts of total

proteins. Total protein extracts from Jurkat and SKBR3 cells were used as positive control of CD147 expression.

**Cyto-ID Autophagy detection.** We used an autophagy detection kit (CYTO-ID Autophagy detection kit, ENZ-51031-K200, Enzo Life Sciences, NY, USA) for monitoring autophagy by flow cytometry analysis in live cells pre-treated with (i) AC-73 (48 hours, 5µM); (ii) ATO (24 hours, ); or (iii) AC-73 and ATO used in combination, as compared to control cells. First, control and pre-treated leukemic cells were seeded in triplicate (15.0 x $10^4$ cells/ml in wells of a 6 wells plate)*;* then cells were harvested and prepared according to manufacturer's instructions prior to analysis by flow cytometry FACScan.

**CD147 siRNA.** Knockdown of CD147 expression by using RNA interference and transient transfection, was performed to silence CD147 gene with CD147-synthetic small interfering RNA (CD147-siRNA; SMARTpool, ON-TARGET plus CD147 from Dharmacon). $CD34^+$ HPCs were transfected with 50 nM CD147-siRNA, or an equal amount of non-targeting control siRNA (c-siRNA; ON-TARGETplus Non-targeting Control siRNA from Dharmacon), by using lipofectamine according to the manufacturer's instruction and as previously described.[29,30] Transfected cells were collected 24 h post-transfection and cultured under both Mo and G liquid culture conditions and for clonogenic assays. At day 2 of Mo and G cultures corresponding at day 3 post-transfection, an aliquot of cells were harvested to assess CD147 silencing by real time PCR analysis of CD147 mRNA knockdown (not shown) and by flow cytometry analysis of CD147 protein level reduction.

**Clonogenic assay and colony formation assay.** Clonogenic assays were performed with (CD147-siRNA)-transfected HPCs as compared to (c-siRNA)-transfected HPCs, in methylcellulose cultures under conditions favoring G and Mo proliferation and differentiation, as previously described.[30]

Colony formation assays were carried out using leukemic cells treated with AC-73 (5 μM) and plated in semisolid medium (methylcellulose) and results were compared to control leukemic cells (500 cell/ml). Cells were maintained in 1 ml of medium Eagle's agar containing serum in presence, or not, of AC-73, at 37°C in a humidified incubator for 14 days. Cell colonies were counted under a microscope using three different plates for each condition.

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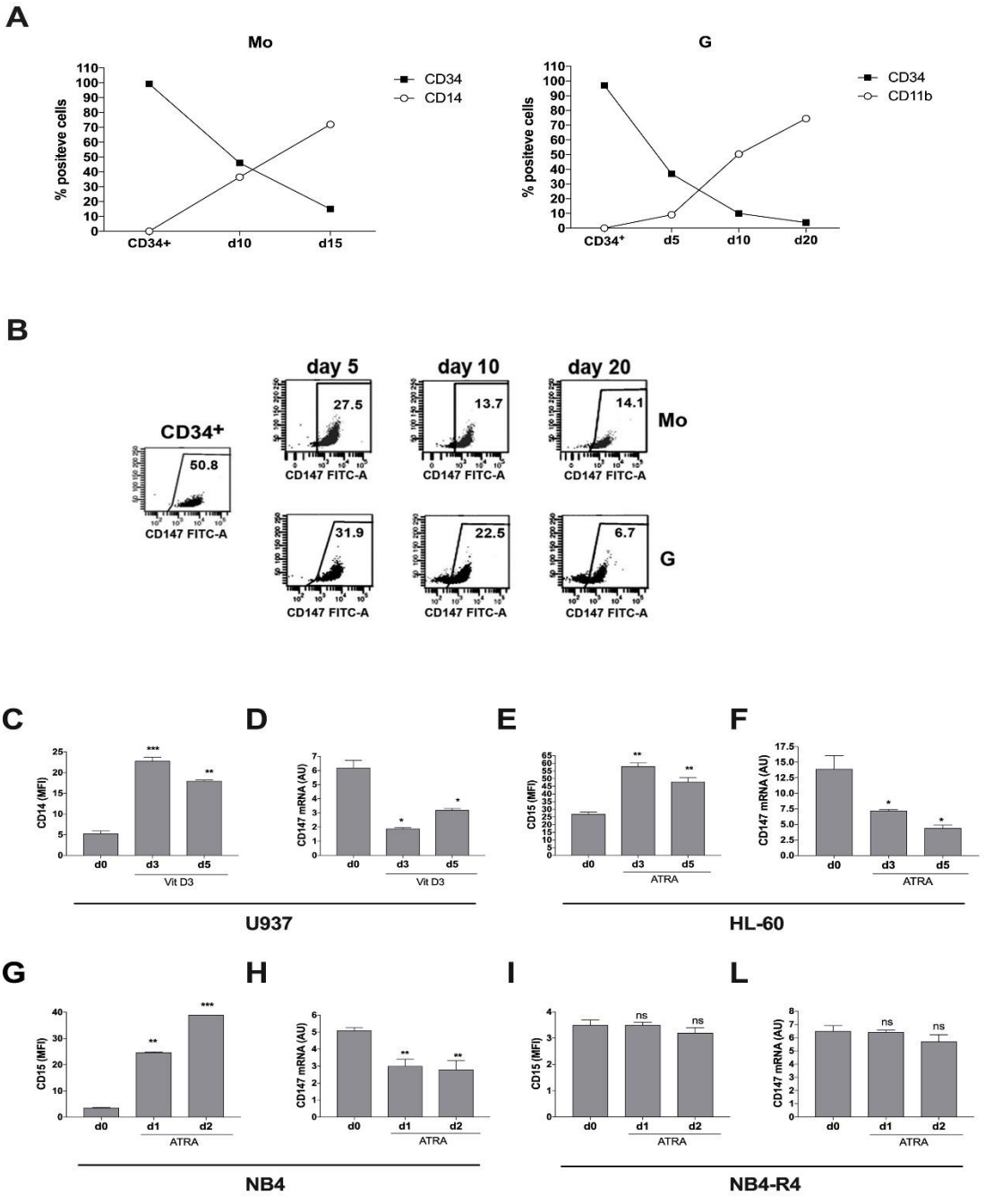
## Supplementary Figures

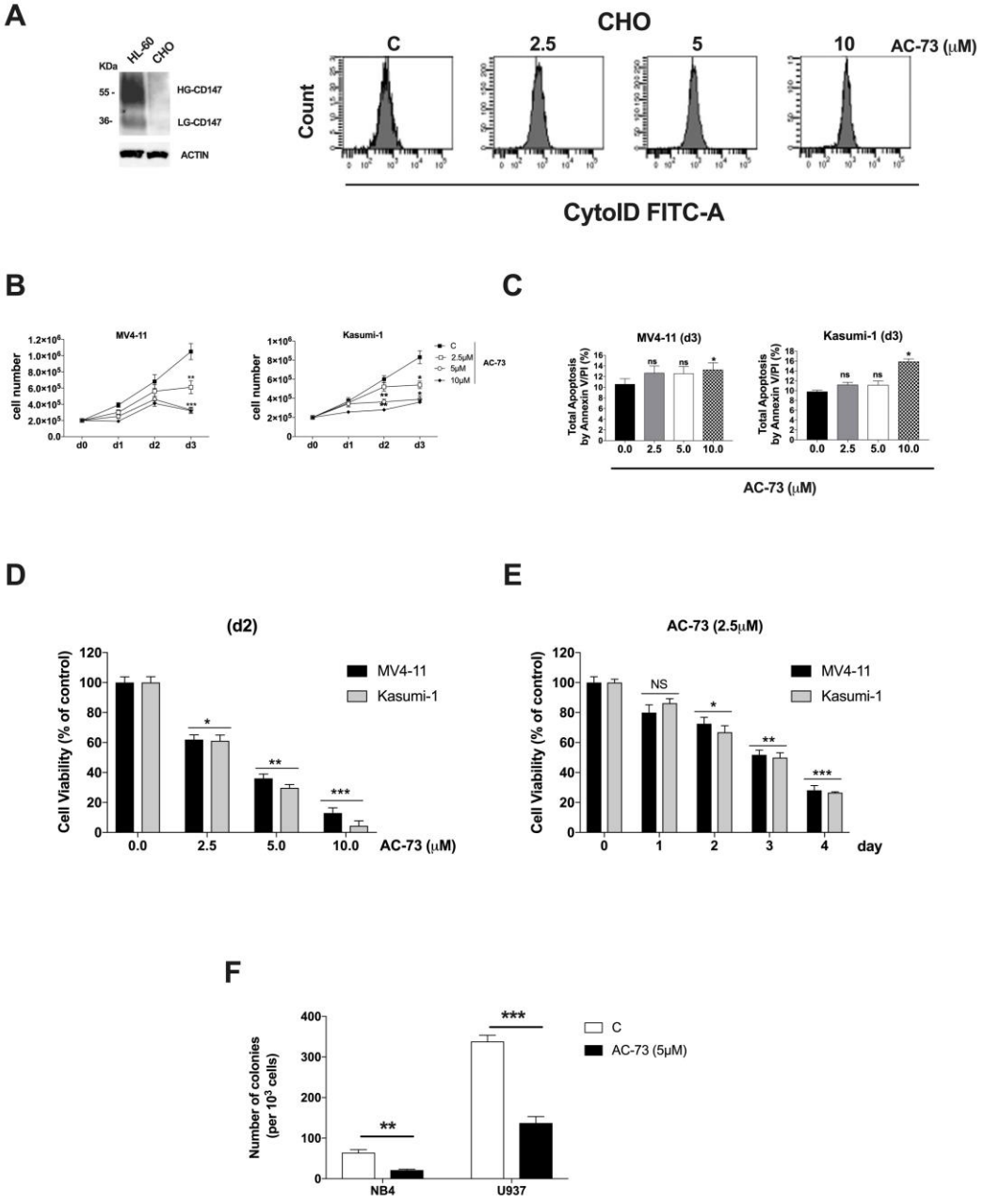
## Supplementary Figure S1.




**Legend Supplementary Figure S1. CD147 expression is downregulated during monocytic or granulocytic differentiation of HPCs and AML cell lines.** (A) Flow cytometry analysis of decreased expression of CD34+ on HPCs, while lineage-specific

markers such as CD14 and CD11b on Mo and G differentiating HPCs respectively, increase in Mo and G liquid phase culture system. One representative experiment is shown. (B) CD147 membrane protein expression decreases during Mo and G differentiation and maturation of HPCs, as shown by flow cytometry analysis. (C) Vit.D3 induces monocytic differentiation of U937 cells, as controlled by flow cytometry analysis of the increase expression of CD14 in these cells. (D) CD147 mRNA expression decreases during vit.D3-induced monocytic differentiation of U937 cells, as shown by qRT-PCR analysis. (E, G) ATRA induces granulocytic differentiation of HL-60 cells and NB4, as controlled by flow cytometry analysis of the increase expression of CD15 in these cells. (F, H) CD147 mRNA expression decreases during ATRA-induced granulocytic differentiation of HL-60 and NB4 cells, as shown by qRT-PCR analysis. (I) NB4-R4 cells are resistant to ATRA treatment, as shown by the low and constant level of CD15 detected by flow cytometry analysis, as compared to NB4 cells. (L) High level of CD147 mRNA cannot decrease in ATRA-resistant NB4-R4 cells, as compared to control (d0) NB4-R4 cells. (A) One representative flow cytometry analysis is shown. (B) Representative flow cytometry plots are shown. (C-L) The result of three independent experiments (mean ± SEM values) is shown; MFI is for Mean Fluorescence Intensity; AU is for arbitrary units; significance is *$p<0.05$; **$p<0.01$; ***$p<0.001$; ns is for not significant.

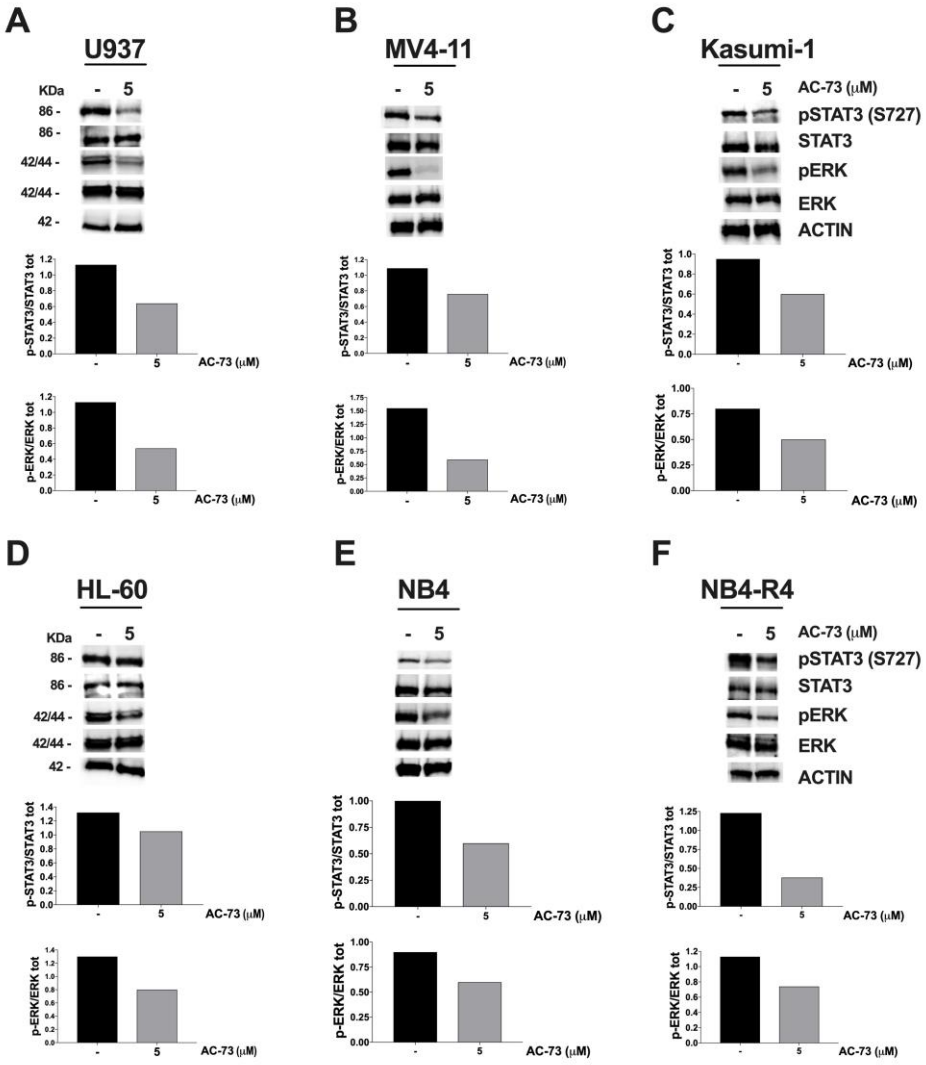
**Supplementary Figure S2.**




**Legend Supplementary Figure S2. Effects of AC-73 treatment on the Chinese hamster ovary cell line (CHO) and on human MV4-11 and Kasumi-1 AML cell lines.** (A) *Left panel* CHO is a negative cell line for CD147 expression, as shown by western blot analysis; *Right panels* dose response analysis of AC-73 treatment on the autophagy flux of CHO cells, as compared to control cells. (B) Dose response analysis of AC-73 treatment on leukemic

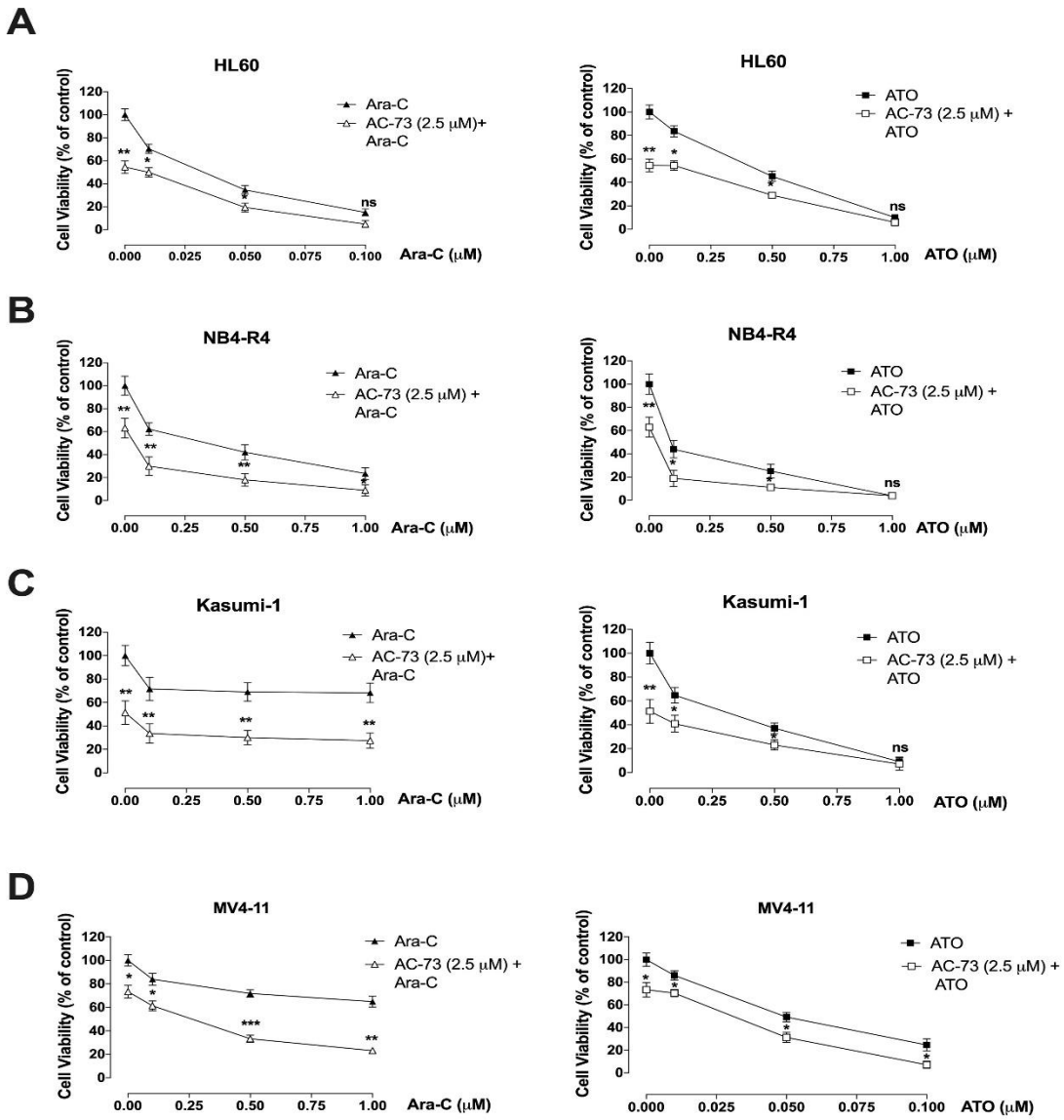
MV4-11 and Kasumi-1 cell growth, as compared to control cells (C). (C, D) Dose response analysis of AC-73 treatment performed on leukemic MV4-11 and Kasumi-1 cell apoptosis (C) and viability (D), as compared to control leukemic cell (0.0). (E) Time course analysis of 2.5 µM AC-73 treatment on leukemic MV4-11 and Kasumi-1 cell viability, as compared to control cells (day 0). (F) Colony formation assays used to detect proliferation, show that AC-73 treatment inhibits the clonogenic growth of leukemic NB4 and U937 cells, as compared to control (C) cells. (A) One representative experiment of three is shown; actin is an internal control. (B-F) The result of three independent experiments (mean ± SEM values) is shown; significance is *$p<0.05$; **$p<0.01$; ***$p<0.001$; ns is for not significant. (C) Total apoptosis by Annexin V/ PI (%), detected by using flow cytometric apoptotic assays, is indicated. (D, E). Data from cell viability assays are presented as percentage viable cell relative to control.

**Supplementary Figure S3**




**Legend Supplementary Figure S3. AC-73 affects ERK1/2 and STAT3 activities in leukemic cells.** (A-F) *Upper panels* Western blot analysis of phospho-STAT3(ser727) (pSTAT3(S727), STAT3, phospho-ERK1/2 (pERK) and ERK1/2 (ERK) proteins expression in leukemic cell lines treated for three days with 5 μM of AC-73, as compared to control leukemic cells (-). Actin is an internal control. Molecular weights (kDa) are indicated (A-F) *Lower panels* Quantification of total and phosphorylated ERK1/2 and STAT3 proteins by densitometry analysis; ratio pSTAT3/STAT3 and pERK/ERK of one representative experiment out of three is shown.

**Supplementary Figure S4.**




**Legend Supplementary Figure S4. AC-73 increases the sensitivity of HL-60, NB4-R4, Kasumi-1 and MV4-11 leukemic cells to chemotherapeutic treatment.** (A-D) Cell viability assays were performed on HL-60, NB4-R4, Kasumi-1 and MV4-11 leukemic cells treated first 24 hours with AC-73alone (2.5 μM), then in combination with Ara-C (AC-73 + Ara-C, left panels) or ATO (AC-73 + ATO, right panels) for 48 hours, as compared to treatment with single drug, AC-73 (in graph indicated as AC-73 + Ara-C or ATO: 0.00 μM), Ara-C and ATO. (A-D) The result of three independent flow cytometry experiments (mean ± SEM values) is shown; significance is *$p<0.05$; **$p<0.01$; ns is for not significant.