SUPPLEMENTARY APPENDIX

# Pharmacological inhibition of dihydroorotate dehydrogenase induces apoptosis and differentiation in acute myeloid leukemia cells

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**The supplementary information includes Supplementary Materials and Methods, and 10 supplementary figures.**

## SUPPLEMENTARY METHODS AND MATERIALS

### Reagents

Isobavachalcone (IBC) was purchased from Sigma-Aldrich (Shanghai, China). Leflunomide (LEF) was obtained from MedChemExpress (Shanghai, China). Antibodies against cleaved PARP, GAPDH, cleaved caspase-3, cleaved caspase-9, p21, MYC were obtained from Cell Signaling Technology (MA, USA). DHODH, OGT and GAPDH antibodies, and anti-rabbit secondary HPR-conjugated antibody were purchased from Proteintech Group (PA, USA). Antibodies (mouse: CD11b; human: CD14, CD33 and CD34) were obtained from BioLegend (CA, USA). CNBr-Sepharose 4B beads were purchased from GE Healthcare (PA, USA). All other reagents were obtained from Sigma-Aldrich unless otherwise specified.

### Protein expression and purification

Human DHODH plasmid was kindly provided by Prof. Jon Clardy (Harvard Medical School).[1] DHODH (Met30-Arg396) was cloned into pET-19b plasmid, and then the recombinant plasmid was transformed into BL21 (DE3) *E.coli* cells for protein expression. Cells were grown at 37 °C in 2×YT medium and protein expression was induced by addition of 0.5 mM isopropyl-D-thiogalactoside (IPTG) at an $OD_{600}$ of 0.6-0.8. All the following steps were at 4 °C. The cells were harvested by centrifugation after induction of 14 h. Subsequently, the cell pellet was lysed with sonication in buffer A (50 mM HEPES, pH 7.7, 300 mM

NaCl, 10% glycerol, 1% Triton X-100). The lysate was centrifuged at 12000 g for 45 min, and the supernatant was incubated with nickel-nitrilotriacetic acid (Ni-NTA) resin (Novagen, Darmstadt, Germany) which was pre-equilibrated with buffer. The column was washed with buffer B (50 mM HEPES, pH 7.7, 300 mM NaCl, 10% glycerol, 1% Triton X-100 and 50 mM imidazole) for several times and eluted with buffer C (50 mM HEPES, pH 7.7, 300 mM NaCl, 10% glycerol, 1% Triton X-100 and 300 mM imidazole). The purified proteins were dialyzed against buffer A and then concentrated by using a 30 kDa centrifugal filter from Millipore (KGaA of Darmstadt, Germany) and stored at -80°C.

**DHODH enzymatic assay**

The enzymatic assay of DHODH was determined using the 2, 6-dichloroindophenol (DCIP) method as described previously.[1] In the assay, compounds were incubated in reaction buffer (50 mM Tris, 120 μM DCIP, 100 μM Coenzyme $Q_0$ ($CoQ_0$), 0.1% Triton X-100, pH 8.0) with DHODH (15 nM) for 10 min at room temperature. The reaction was initiated by addition of 500 μM DHO). The enzyme activity was monitored by recording the reduction of DCIP in the absorption at 600 nm over the course of 6 min using the microplate reader Synergy 2 (BioTek, VT, USA). The half maximal inhibitory concentration ($IC_{50}$) was determined from the results of at least three independent tests and calculated by the GraphPad5 (CA, USA).

**Enzymatic kinetic analysis**

To character the inhibitor type, Lineweaver-Burk analysis were performed as described previously.[2] The reaction buffer contained 50 mM Tris (pH 8.0) and 150 mM KCl. The concentrations of $CoQ_0$ (10-400 μM) and DHO (10-400 μM), as well as the IBC (0-200 nM), were varied appropriately for each experiment. The result was analyzed by monitoring the production of $CoQ_0$ at 287 nm using a Hitachi U-2000 spectrophotometer (Tokyo, Japan). All experiments were performed at 25 °C. The experiment was carried out at least three times.

**Thermal shift assay**

Thermal shift assay was performed by the CF×96TM Real Time System (Bio-Rad, CA, USA) as described in our previously study.[3] In brief, SYPRO orange stock solution (5000X, Invitrogen, NY, USA) was diluted 1:1000 in the assay buffer (25 mM HEPES, 150 mM NaCl, pH 7.5) and then DHODH protein was added to a final concentration of 5 μM. Then, the mixed sample was incubated with indicated compounds or DMSO in the 96-well iCycler iQ PCR plate (Bio-Rad, CA, USA). The plate was heated from 25 to 79.6 °C at a heating rate of 0.3 °C /18 s. The fluorescence intensity was measured every 30 seconds with Excitation/Emission: 492/610 nm. The data was calculated by fitting the Boltzmann equation to the sigmoidal curve as described. The experiments were carried out at least three times.

**Nuclear Magnetic Resonance (NMR) spectroscopy analysis**

One-dimensional NMR data were acquired on a Bruker Avance III-600 MHz NMR spectrometer equipped with a cryogenically cooled probe (Bruker biospin, Germany) at 25 °C.[4] Ligand observed CPMG NMR experiments were applied to analyze ligand-protein interactions. Samples of experiment contained 200 μM IBC and 200 μM IBC in addition of 2.5 μM or 5 μM DHODH protein. Samples were diluted in $D_2O$ buffer (20 mM $NaH_2PO_4/Na_2HPO_4$, 100 mM NaCl, pH 7.4, 5% DMSO) and then used in NMR data acquisition.

**Isothermal Titration Calorimetry (ITC) assay**

The binding affinity between DHODH and IBC was directly measured using an iTC200 instrument (Microcal, GE Healthcare, PA, USA) at 25 $^{o}$C.[5] The experiment was performed in an ITC buffer containing 50 mM HEPES, 300 mM NaCl, 10% Glycerol, 0.1% Triton. The stock solutions of IBC and recombinant DHODH protein was dissolved in the ITC buffer at the concentrations of 200 μM and 25 μM before titrations, respectively. The final concentration of DMSO in the titration buffer is less than 5% of the total volume. All titrations of IBC into DHODH were performed using an initial injection of 0.4 μl followed by 19 identical injections of 2 μl with a duration of 4 seconds per injection and a spacing of 120 seconds between injections. The heat was obtained by injecting IBC into the DHODH. The experimental data was processed by the supplied MicroCal Origin software package (PA, USA). The thermodynamic parameters

were calculated with the formula $\Delta G = \Delta H - T\Delta S = -RT \ln K$, where T, R, K, ΔG, ΔH and ΔS represent the experimental temperature, the gas constant, binding constant, the changes in free energy, the changes in enthalpy and entropy of binding, respectively.

**Molecular docking simulation**

In order to investigate the binding mechanism, we employed molecular docking to analyze the interaction between IBC and DHODH. The crystal structure of DHODH in complex with a synthetic inhibitor NO.33 (PDB code: 4YLW) was derived from Protein Data Bank. Molecular docking simulation was carried out using the AutoDock Vina (The Scripps Research Institute, CA, USA).[6] All water molecules and ligands were removed before molecular dynamics simulation, and then IBC was docked into the active site of DHODH. The best-scored complex conformation of IBC and DHODH was chosen by the scoring function to estimate the binding scaffold. All figures were generated by PyMOL software (https://www.pymol.org/).

**Cell culture**

HL60 and THP1 cells were purchased from the Chinese Academy of Sciences (Shanghai, China). U937 and MOLM-13 cells were purchased from American Type Culture Collection (Manassas, VA). HL60/ADR, an Adriamycin (ADR)-resistant cell line, was obtained from the Institute of Haematology, Chinese

Academy of Medical Sciences (Tianjin, China). All cells were cultured in RPMI 1640 medium (Hyclone, NY, USA) supplemented with 10% (v/v) FBS (Gibco, NY, USA) and 1% penicillin/streptomycin (Life Technologies, CA, USA) in a humidified atmosphere with 5% $CO_2$ at 37°C. ADR (0.5 μg/ml) was added to the medium to maintain the drug-resistance phenotype in the HL60/ADR cells. All cell lines have been authenticated by Short Tandem Repeat (STR) profiling, and characterized by mycoplasma detection.

**Cell proliferation assay**

Cells were seeded into 96-well plates for 12 h, and then treated with indicated concentration of compounds. After varied times of incubation, CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) solution (Progema, WI, USA) was added and incubated for another 3 h, and then the optical density was measured at 490 nm using microplate reader Synergy 2 (BioTek, VT, USA). All experiments were carried out at least three times.

**Uridine rescue *in Vitro***

Uridine was dissolved in DMSO at a concentration of 100 mM and stored at -20 °C. Cells were pretreated with uridine (200 μM) for 12 h, and then indicated concentrations of IBC was supplemented into the cells.

**Real-time PCR assay**

Total RNAs were isolated using Trizol reagent (Invitrogen, NY, USA) according to the manufacturer's instructions. RNAs were reversed transcribed into cDNA using the ReverTra Ace Qpcr RT Master Mix (Toyobo, Osaka, Japan). PCR reaction was performed as follows: 95 °C for 2 min, and followed by 35 cycles of amplification (95 °C for 20 s, 58 °C for 30 s and 72 °C for 40 s, with a final extension of 72 °C for 10 min. The PCR products were calculated by DNA gel electrophoresis analysis with the GAPDH for normalization. The sense and antisense primers for *MYC* were 5'-GGCTCCTGGCAAAAGGTCA-3' and 5'-CTGCGTAGTTGTGCTGATGT-3', respectively. The sense and antisense primers for *p21* were 5'-TGTCCGTCAGAACCCATGC-3' and 5'-AAAGTCGAAGTTCCATCGCTC-3', respectively. The sense and antisense primers for GAPDH were 5'-CTTCACCACCATGGAGGAGGC-3' and 5'-GGCATGGACTGTGGTCATGAG-3', respectively.

**Western blot analysis**

Cells and tumor tissues were harvested, washed with cold PBS, and then lysed with RIPA Lysis Buffer with 1 mM phenylmethylsulfonyl fluoride (PMSF). The total protein concentrations were measured using a standard BCA protein assay kit (Bio-Rad, CA, USA) according to the manufacturer's manual. Samples were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then blotted onto a polyvinylidene difluoride (PVDF; EMD Millipore, Darmstadt, Germany) membrane on ice. After transfer,

the membranes were probed with specific primary antibodies (1:1000) at 4 °C overnight. The specific protein bands were detected using a chemiluminescence reagent after hybridization with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000).

**Hematoxylin** & **Eosin (HE) staining**

The indicated tissues were fixed in 4% formaldehyde solution and then embedded in paraffin. Sections were cut at 5 μm and stained with Haematoxylin & Eosin (HE). Histology images were acquired using a Ti-S bright field microscope (Nikon, Tokyo, Japan).[7]

**Trypan blue staining assay**

HL60 cells ($2 \times 10^5$) were treated with various concentration of IBC for 24, 48, and 72 h, respectively. Cell counting was performed by trypan blue staining method using Cellometer Mini Cell Counter (Nexcelom, Bioscience, USA). Dead cells were stained as blue whereas living cells were unstained. The experiments were repeated three times.

**Luciferase reporter assay**

293T cells were seeded into 24-well plates and grown overnight, then cells were transfected with a *MYC*-luc reporter (addgene ID: 16601) and pRSVluc plasmid (as an internal control) using Lipofectamine2000 according to the

manufacturer's instruction. Cells were incubated with different concentrations of compound for 24 h after transfection. Luciferase activity was subsequently measured 24 h after compound incubation. The reporter activity was normalized against *Renilla* luciferase activity.

***In vitro* pull-down assay with IBC-Sepharose 4B Beads.**

The IBC-Sepharose 4B suspension was prepared as described previously.[8,9] CNBr-Sepharose 4B powder (0.3 g) was suspended in 1 mM HCl solution, then washed with coupling buffer (0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl). Then, 2 mg IBC was added to the solution and stirred at 4 °C overnight. Finally, Sepharose 4B conjugated with IBC was washed with the coupling buffer for seven times, the solution was replaced with PBS and stored at 4 °C. For the *in vitro* and *ex vivo* pull-down assays, recombinant DHODH (500 ng) or HL60 cell lysates (600 μg) incubated with IBC-Sepharose 4B (or Sepharose 4B alone as a control) beads in reaction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.01% NP-40, 1mM DTT, and 1 mg/ml bovine serum albumin) and mixed on a rotary shaker at 4 °C overnight. After then, the beads were washed 5 times with washing buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.01% NP-40, 1 mM DTT). The binding proteins were visualized by Western blotting.

**Combination Studies in vitro**

To explore the anti-proliferation effect of IBC in combination with ADR,

combination index (CI) versus fractional affect (Fa) were calculated using CalcuSyn software Version2.1 (Biosoft, Cambridge, UK) to analyses the interaction (<1: synergistic, =1: additive or >1: antagonistic) according to the method.[10] In brief, AML cells were incubated with increasing concentration of IBC and ADR alone or in combination for 48 h. Then the cell viability was measured by MTS assay, the CI and Fa values for the combination of IBC and ADR were calculated using CalcuSyn software Version 2.1.

**Cellular thermal shift assay (CETSA)**

The cellular thermal shift assay was performed according to the method as described previously.[11] Briefly, HL60 cells were incubated with DMSO or 30 μM IBC for 12 h, then cells were collected, washed and resuspended in phosphate-buffered saline (PBS). Each sample ($3\times10^6$ cells, 50 μl) subsequently were heated at addicted temperatures for 3 min on PCR instrument (Bio-Rad, CA, USA) and frozen with liquid nitrogen twice. The supernatants were analyzed by Western blot.

**Analysis of intracellular uridine**

HL60 cells were treated with DMSO or 30 μM IBC for 48 h. The cells were washed three times in normal saline. For metabolite extraction, cells ($1.0 \times 10^7$) were resuspended in ice-cold 80% (v/v) methanol, and then lysed by repeated freezing and thawing with liquid nitrogen. Subsequently, the lysate was

centrifuged at 12000 g for 30 min, and the supernatant was collected. Theophylline was used as an internal standard. Metabolites were analyzed by an Agilent 1290 LC system coupled with a 6460 triple-quadrupole mass spectrometer (Agilent Technologies, USA). The raw data were acquired and processed with Agilent MassHunter version 5.0.280.1 software (Agilent Technologies, USA). The experiments were repeated three times. The details can be found in a previous study.[12]

**Subcutaneous leukemia mode**

The experimental protocol was conducted as described previously.[13] On the day of injection, HL60 or HL60/ADR cells ($1.5 \times 10^7$) were suspended in PBS (200 μl) on ice, and were then implanted subcutaneously in the left bilateral flanks of 6-8 weeks old BALB/c nude female mice, respectively. The mice were randomly assigned into groups (8 mice/group) when the volumes of solid tumor reach ～100 $mm^3$ and then treated with indicated dose of compounds for 18 days. IBC and LEF were oral administered once a day, while the ADR was administered once every 3 days by i.p. injection. Mice were weighed every 3 days. Tumor size was monitored every 3 days using calipers and calculated according to a standard formula: $V = 0.52*(L \times W^2)$. L stands for length and W is width.

**Intravenous leukemia model**

Six-weeks-old NOD-SCID female mice were irradiated for 12 hours (totally 2.0 Gy) before i.v. injection of HL60 cells. Cells ($1.2 \times 10^7$) were dissolved in RPMI1640 medium and then implanted by tail vein injection. 7 days after transplantation, the mice were treated with IBC, ADR or drug combination. No animals were excluded from analysis. The details can be found in a previous study. [12]

**Fluorescence assay of DHODH activity in tumor tissues**

DHODH activity in tumor tissue was measured by a recently developed fluorescence assay .[14] Tumor samples were shredded and lysed in sterile water by sonication for 15 min on ice. The lysates were obtained at 15000 g for 30 min. Then lysates were incubated in buffer (200 mM $K_2CO_3$-HCl, 0.2% triton-x100, 500 μM DHO and 100 μM $CoQ_0$ pH 8.0) at 37 °C for 1 h. Subsequently, the mixture solution was added into the reaction buffer (4 mM 4-trifluoromethylbenzamidoxime, 8.0 mM $K_3[Fe(CN)_6]$, 80 mM $K_2CO_3$-HCl, PH 8.0), and the reaction was performed by heating at 80 °C for 5 min. The reaction was stopped by cooling on ice and the fluorescence intensity was measured with a PT1-QM4 steady-stead fluorimeter (PTI, USA) at maximum excitation and emission wavelengths of 340 and 460 nm, respectively.

**Bioinformatics analyses**

We download the microarray data and survival profiles on 242 samples of bone

marrow or peripheral blood mononuclear cells from adult patients with untreated acute myeloid leukemia (GSE12417).[15] Patients were grouped into top 20% lowly expressed (blue) vs. top 20% highly expressed (red) groups based on the normalized expression level. The P-value of Kaplan-Meier survival analysis was performed using a log-rank test in the GraphPad Prism 5. We downloaded gene expression data in cancer cell lines and AML patients from the Cancer Cell Line Encyclopedia (CCLE)[16] and The Cancer Genome Atlas (TCGA) dataset[17] respectively. P-value in gene differential expression analysis was calculated using Wilcoxon one-tailed test in R software (v3.2.3, www.r-project.org).

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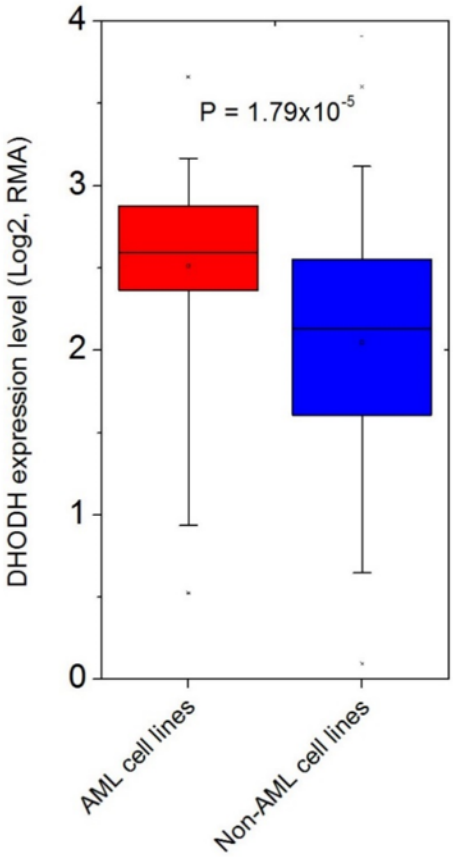
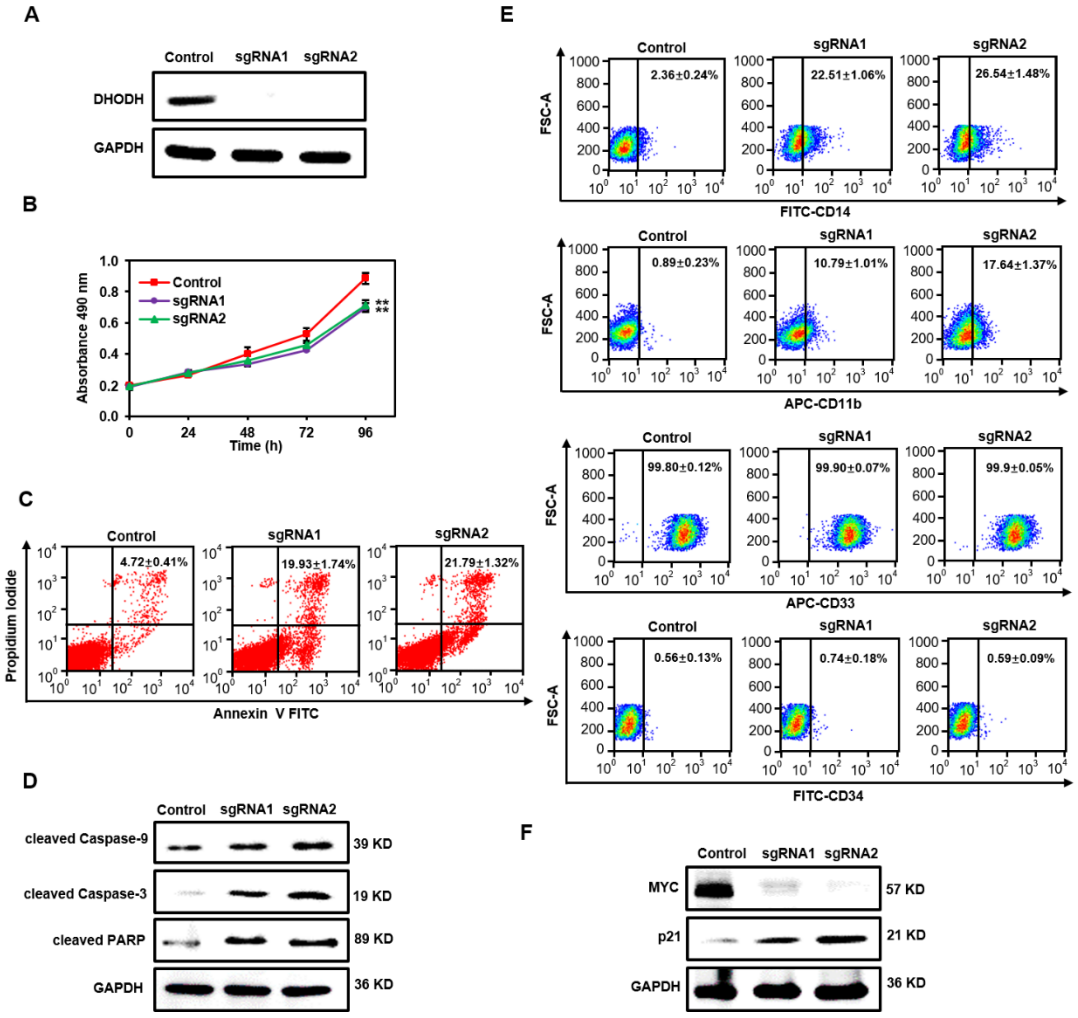
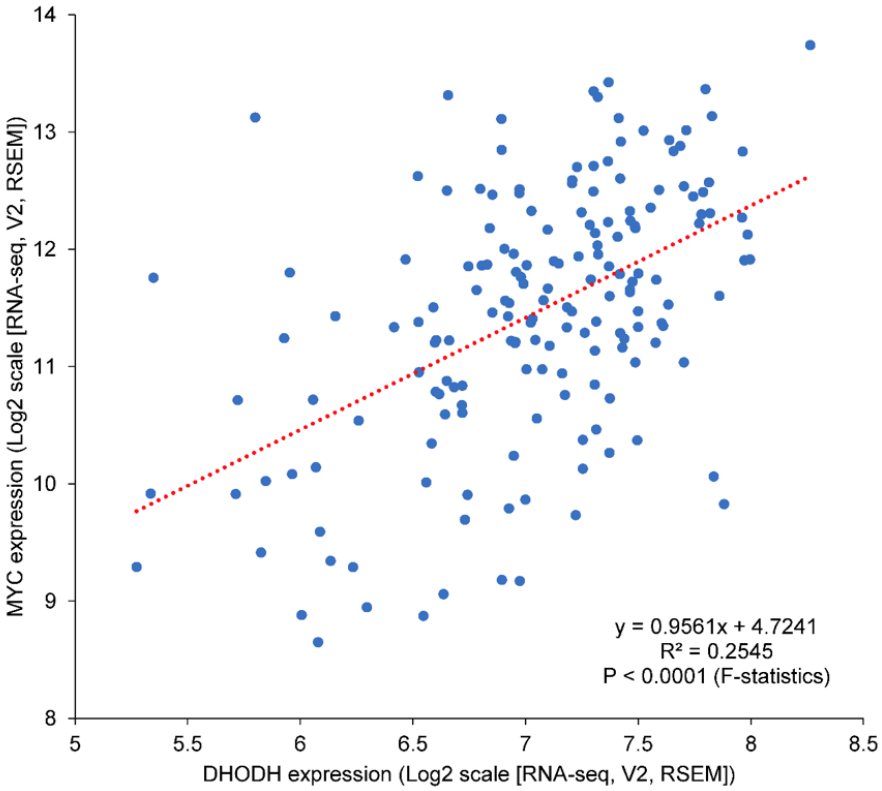
## Supplementary Figures



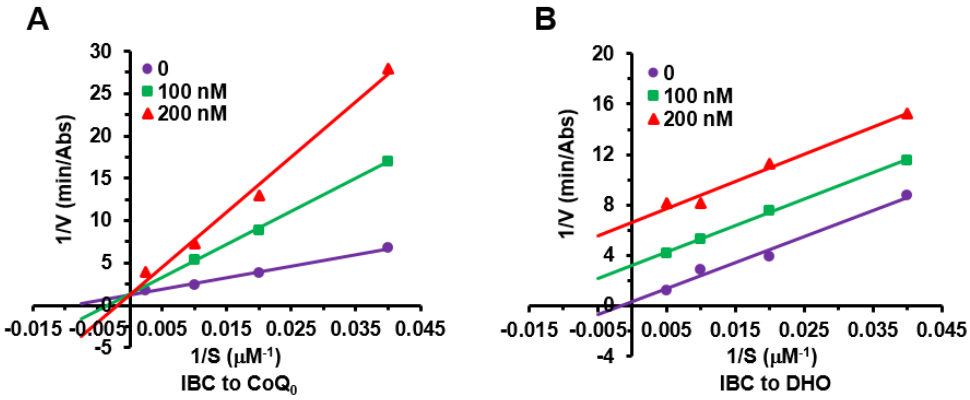

**Supplementary Figure S1. Gene expression level of DHODH in 32 acute myeloid leukemia (AML) cell lines compared to 987 non-AML cell lines.** Gene expression data in cancer cell lines were downloaded from the Cancer Cell Line Encyclopedia (CCLE) (15). P-value was calculated using Wilcoxon one-tailed test in R software (v3.2.3, www.r-project.org). *DHODH* is highly expressed in AML cell lines compared to non-AML cell lines.



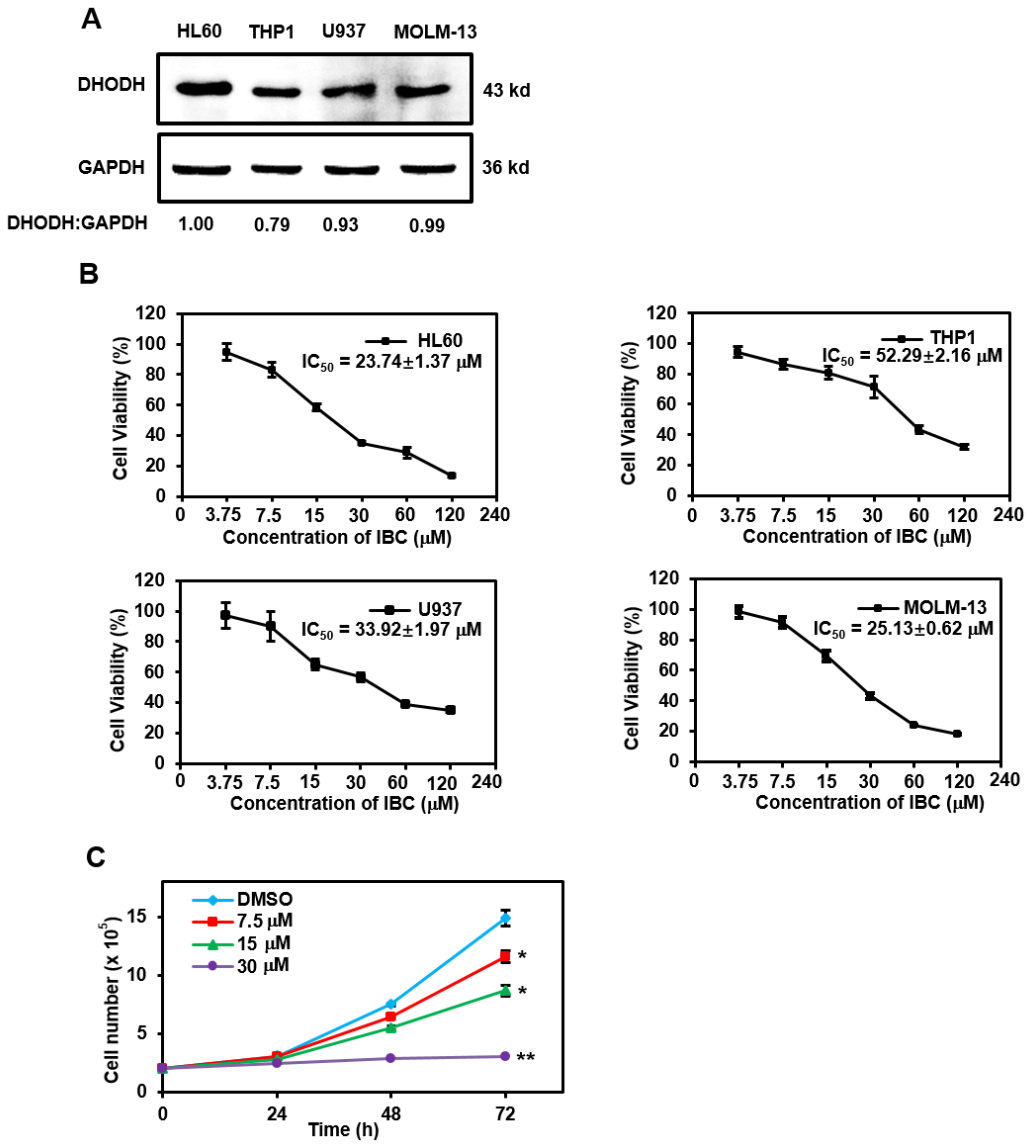

**Supplementary Figure S2. Knockout of DHODH result in THP-1 cell apoptosis and differentiation. (A)** Knockout of DHODH in THP1 cells were analyzed by western blot. **(B)** Knockout of DHODH suppressed the growth of THP1 cells. The cell growth was evaluated by MTS assay at 24 h intervals up to 96 h through three independent experiments. Bar groups represent the mean ± S.D. Student's t-test was performed, $^{**}P < 0.01$. **(C)** DHODH knockout results in apoptosis in THP1 cells. Cell apoptosis were analyzed by flow cytometry at 96 h after infection. **(D)** The expression levels of apoptosis-related proteins in THP1 cells were detected by western blot. **(E)** Flow cytometry demonstrates upregulation of cell surface markers CD14 and CD11b, whereas no effect on CD33 and CD34, after knockout of DHODH in THP1 cells. Cells were measured at 96 h after infection. Data represent mean ± S.D of three independent experiments. **(F)** Knockout of DHODH resulted in reduced expression of MYC and upregulated expression of p21 in THP1 cells.



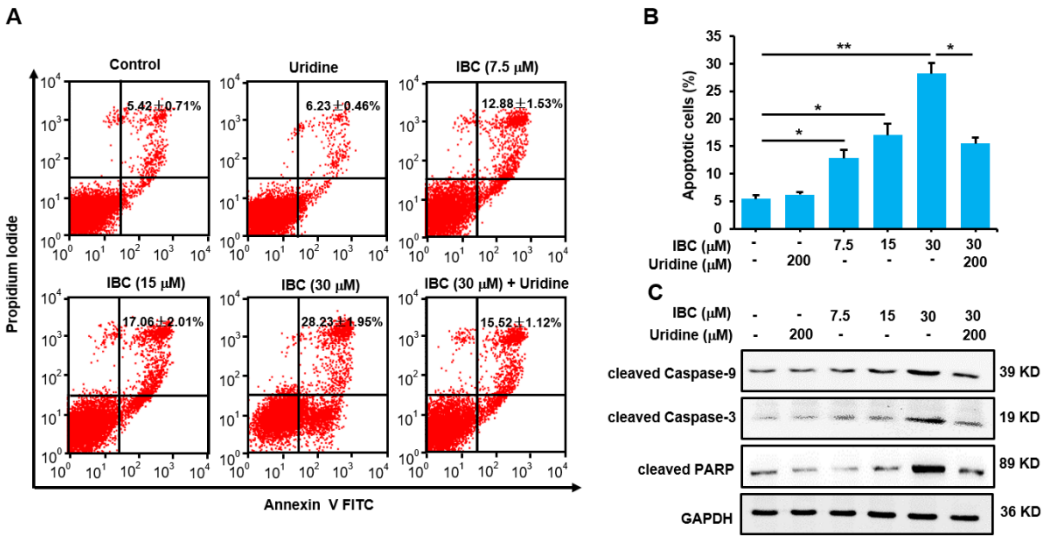

**Supplementary Figure S3. Gene co-expression analysis between *DHODH* and *MYC* in 173 acute myeloid leukemia patients.** Gene expression data (RNA-seq, V2, RSEM) in AML patients were downloaded from The Cancer Genome Atlas (TCGA) dataset (16). *DHODH* is highly co-expressed with *MYC* in AML patients.



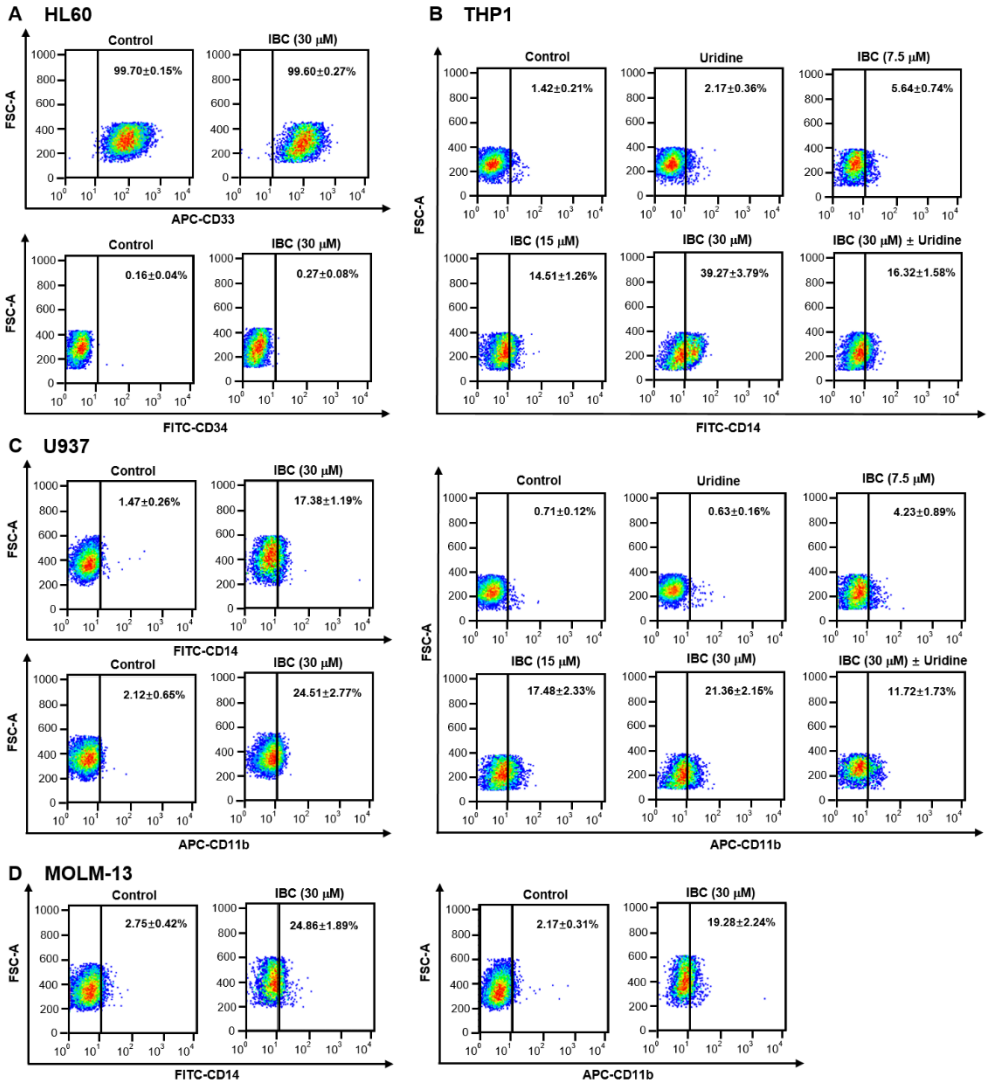

**Supplementary Figure S4. Inhibitor type of IBC against DHODH.** IBC acted as a $CoQ_0$ competitive inhibitor (**A)** and an uncompetitive inhibitor versus substrate DHO **(B)**.



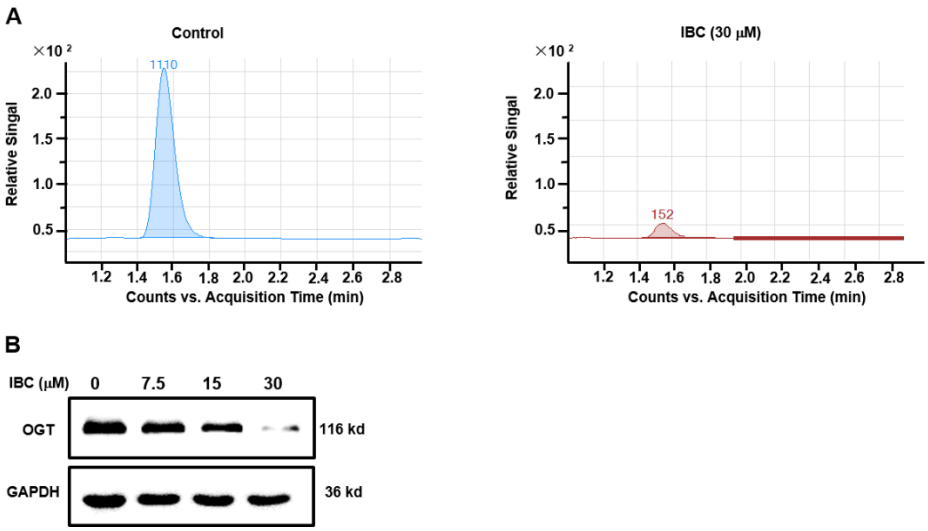

**Supplementary Figure S5. The effect of IBC on AML cells survival. (A)** Western blot analysis the expression levels of DHODH in different AML cell lines (HL60, THP1, U937 and MOLM-13). **(B)** AML cells were treated with increasing concentration of IBC for 48 h, and cell viability was measured by MTS assay. **(C)** HL60 cells were treated with different concentrations of IBC for 24, 48 and 72 h, and then counted using trypan blue exclusion. Data represent mean ± SD of three independent experiments. Student's t-test was performed, **P* < 0.05, ***P* < 0.01.



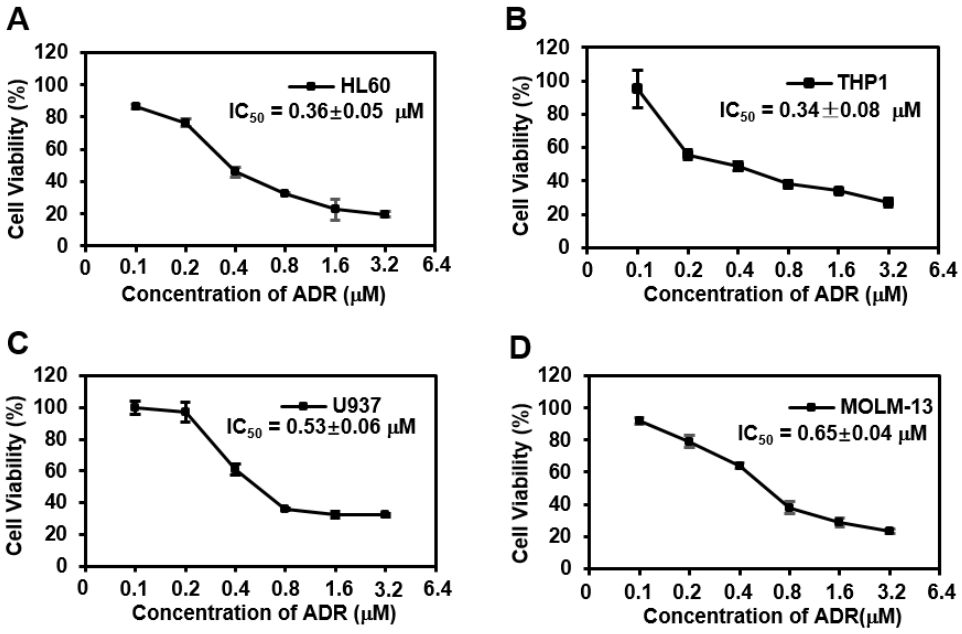

**Supplementary Figure S6. The effect of IBC on THP1 cell apoptosis. (A)** THP1 cells were treated with IBC at the indicated concentrations for 72 h. Cell apoptosis was detected by Flow cytometry using AnnexinV-FITC/PI double stained. **(B)** The quantitative data of cell apoptosis in **(A)**. **(C)** Changes in apoptosis-related proteins after IBC or uridine treatment for 72 h. Data represent mean ± SD.



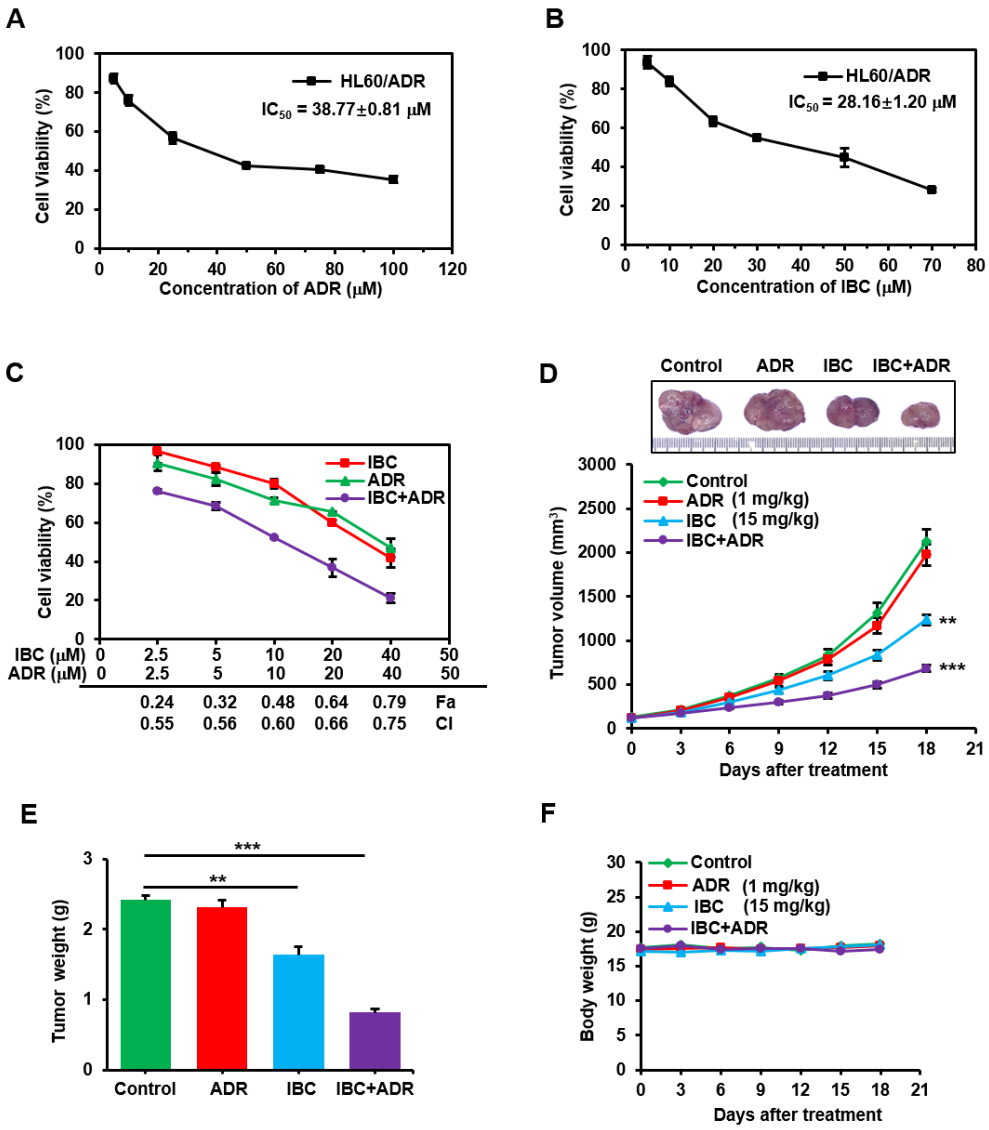

**Supplementary Figure S7. The effect of IBC on AML cell lines (HL60, THP1, U937 and MOLM-13) differentiation. (A)** HL60 cells were treated with IBC for 72 h, CD33 (left) and CD34 (right) expression were detected by Flow cytometry analysis. **(B)** THP1 cells were treated with different concentration of IBC for 72 h, CD14 and CD11b expression were detected by Flow cytometry analysis. **(C)** U937 cells were treated with IBC for 72 h, CD14 and CD11b expression were detected by Flow cytometry analysis. **(D)** MOLM-13 cells were treated with IBC for 72 h, CD14 and CD11b expression were detected by Flow cytometry analysis. Data represent mean ± SD.




**Supplementary Figure S8. The effect of IBC on uridine and OGT. (A)** Treatment of HL60 cells with IBC results in a depletion of uridine. **(B)** The expression of OGT was analyzed by Western blot at 72 h after treated with increasing concentration of IBC.




**Supplementary Figure S9. The effect of ADR on AML cell lines (HL60, THP1, U937 and MOLM-13).** AML cells were treated with increasing concentration of IBC for 48 h, and cell viability was measured by MTS assay. Data represent mean ± SD.




**Supplementary Figure S10. Synergistic effects of IBC in combination with ADR to HL60/ADR *in vitro* and *in vivo*.** **(A)** HL60/ADR cells showed resistance to ADR. Cell proliferation of HL60/ADR were determined by MTS assay after incubation with increasing concentrations of ADR for 48 h. **(B)** IBC suppressed the growth of HL60/ADR cells in a dose-dependent manner. HL60/ADR cells were treated with various concentrations of IBC for 48 h. **(C)** Synergistic effects of IBC-ADR combination. HL60/ADR cells were incubated with several ratios combination of IBC-ADR for 48 h. The combination index (CI)

calculation was performed by using CalcuSyn software (Version 2.1; Biosoft). Drug combination with CI<1 was considered to be synergistic. **(D)** *Synergistic* effects of IBC and ADR combination therapy in the HL60/ADR xenograft model. Mice with established tumors (n = 8 per group) were treated for 18 days with vehicle, IBC, ADR or drug combination of IBC and ADR. **(E)** Effect of IBC and ADR on tumor weights. **(F)** The body weight of mice treated with vehicle, IBC and ADR were measured for 18 days. No significant changes were observed during the experiments. Data represent mean ± SD. One-way ANOVA test was performed, $^{**}P < 0.01$ and $^{***}P < 0.001$.