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

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ABSTRACT

Acute myeloid leukemia is a disorder characterized by abnormal differentiation of myeloid cells and a clonal proliferation derived from primitive hematopoietic stem cells. Interventions that overcome myeloid differentiation have been shown to be a promising therapeutic strategy for acute myeloid leukemia. In this study, we demonstrate that CRISPR/Cas9-mediated knockout of dihydroorotate dehydrogenase leads to apoptosis and normal differentiation of acute myeloid leukemia cells, indicating that dihydroorotate dehydrogenase is a potential differentiation regulator and a therapeutic target in acute myeloid leukemia. By screening a library of natural products, we identified a novel dihydroorotate dehydrogenase inhibitor, isobavachalcone, derived from the traditional Chinese medicine *Psoralea corylifolia*. Using enzymatic analysis, thermal shift assay, pull down, nuclear magnetic resonance, and isothermal titration calorimetry experiments, we demonstrate that isobavachalcone inhibits human dihydroorotate dehydrogenase directly, and triggers apoptosis and differentiation of acute myeloid leukemia cells. Oral administration of isobavachalcone suppresses subcutaneous HL60 xenograft tumor growth without obvious toxicity. Importantly, our results suggest that a combination of isobavachalcone and adriamycin prolonged survival in an intravenous HL60 leukemia model. In summary, this study demonstrates that isobavachalcone triggers apoptosis and differentiation of acute myeloid leukemia cells via pharmacological inhibition of human dihydroorotate dehydrogenase, offering a potential therapeutic strategy for acute myeloid leukemia.

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Introduction

Acute myeloid leukemia (AML) is a malignant disorder characterized by differentiation and abnormal growth of hematopoietic stem or progenitor cells.[1] AML is typically associated with a rapid onset of symptoms attributed to bone marrow

failure, and may be fatal within weeks or months without treatment.[1] AML is the most common acute leukemia, with an estimated incidence of about 19,000 cases in 2018 in the United States (USA) and a 5-year relative survival rate of approximately 60% in a population of children in the USA, based on data from 2007 to 2013.[2] The major therapeutic paradigm for AML in the past several decades has been either chemotherapy with an anthracycline/cytarabine combination or allogeneic stem cell transplantation.[3] Although traditional chemotherapy induces remission in *de novo* AML patients, only 20-30% of patients survive disease free in the long-term because of the high toxicity of chemotherapeutics, subsequent relapses, and development of drug resistance.[4,5] On the other hand, although targeted therapies, such as the isocitrate dehydrogenase 2 (IDH2) inhibitor enasidenib and the FMS-like tyrosine kinase 3 (FLT3) inhibitor midostaurin, were recently approved by the USA Food and Drug Administration for AML treatment,[6] only relatively few AML patients with actionable mutations of IDH2 or FLT3 will benefit from these drugs.[7-9] Development of innovative therapeutic agents is, therefore, a pressing need to improve the clinical efficacy and quality of life for AML patients.

The human dihydroorotate dehydrogenase (DHODH) enzyme belongs to the class 2 DHODH family. It is anchored at the inner mitochondrial membrane.[10] As an essential enzyme that catalyzes dihydroorotate to orotic acid, DHODH plays a critical role in the *de novo* pyrimidine biosynthesis of DNA and RNA.[11] Rapidly proliferating cells, such as cancer cells and lymphocytes, mainly depend on *de novo* pyrimidine biosynthesis to support their growth rate, indicating that this enzyme is a potential target in the treatment of cancer and autoimmune diseases.[10] A previous study suggested that DHODH is required for rapid proliferation of tumor cells, playing an important role in tumorigenesis and tumor development.[12] Using a unique Homeobox A9-driven leukemia model, Sykes *et al.* recently found that DHODH is a novel metabolic target in differentiation therapy of AML.[13,14] Their pioneer work offers a potential differentiation treatment strategy for patients with AML.[13,14] Several DHODH inhibitors, such as brequinar, have already been evaluated in various clinical cancer trials, but severe adverse reactions limit their clinical application.[15,16]

In this study, we show that CRISPR-Cas9-mediated knockout of DHODH greatly impairs growth, increases apoptosis, and induces differentiation of two AML cell lines, HL60 and THP-1, indicating once again that DHODH is a potential therapeutic target. We identified a novel, direct inhibitor of DHODH, isobavachalcone, by screening a library of natural products. We demonstrate that isobavachalcone effectively triggers apoptosis and induces differentiation in AML cells via direct inhibition of DHODH. Furthermore, our results suggest that isobavachalcone, alone or in combination with adriamycin, significantly prolongs survival in an intravenous HL60 leukemia model.

Methods

The *Online Supplement* contains detailed information on the experimental materials and methods.

All animal care and experimental procedures in this study complied with the protocol approved by the Animal Care and Use Committee at East China University of Science and Technology.

Knockout of dihydroorotate dehydrogenase in HL60 and THP-1 cells

The guide RNA sequences targeting DHODH were designed and cloned into a LentiCRISPRv2 construct (Addgene, #52961).[17] This construct along with psPAX2 (Addgene, #12260) and pMD2.G (Addgene, #12259) helper constructs were co-transfected into HEK-293T cells using Lipofectamine 2000 reagent (Invitrogen, NY, USA) to produce lentiviral supernatants. Viral production was subsequently concentrated 60X by ultracentrifugation. Cells were infected with lentiviral supplemented with polybrene (8 μg/mL) in 24-well plates and infected cells were selected in medium containing puromycin (0.8 μg/mL). The knockout efficiency of single guide RNA (sgRNA) was determined by western blot analysis. The sgRNA targeting exon 2 of human DHODH were listed as follows: sgRNA1: 5'-TTCTTCGACATTGCCGTCGA-3'; sgRNA2: 5'-ACAAGGTCCCAAAGACAG-3'.

Cell apoptosis assay

Cells were seeded into six-well plates and incubated with the indicated concentrations of compounds. The apoptosis assays were performed using an AnnexinV-FITC Apoptosis Detection kit (eBioscience, MA, USA) according to the instructions.[18] The apoptotic cells were analyzed using a BD FACS Calibur flow cytometer (BD Biosciences, NJ, USA).

Differentiation marker analysis

Cultured cells were harvested and washed with phosphate-buffered saline on ice, then resuspended in FACS buffer (phosphate-buffered saline, pH 7.4, supplemented with 1 mM ethylenediaminetetraacetic acid and 2% fetal bovine serum).[19] Antibodies of differentiation markers (CD11b, CD14, CD33 and CD34) were added and incubated for 1 h at 37°C in the dark. Flow cytometer data were collected and analyzed on a BD FACS Caliber using Cell Quest software (BD Biosciences, NJ, USA).

Wright-Giemsa staining

Cells were harvested, washed with phosphate-buffered saline and fixed with 95% ice-cold methanol for 30 min at 4°C.[14] The cells were then seeded on the slide and allowed to dry in the air. Next, the cells were stained with Wright-Giemsa for 5 min and rinsed in deionized water. Finally, coverslips were fixed with Permount prior to microscopy (Nikon, Tokyo, Japan).

Statistical analysis

All values are expressed as the mean ± standard deviation of at least three independent experiments. GraphPad Prism 5.0 software (GraphPad software, CA, USA) was used for the statistical analysis. Comparisons between two groups were analyzed using the two-tailed Student *t*-test. For multiple comparisons, one-way ANOVA followed by Tukey multiple comparison tests were performed. P values < 0.05 are considered statistically significant.

Results

Dihydroorotate dehydrogenase overexpression is associated with poor prognosis in acute myeloid leukemia

We examined the relationship between DHODH expression and overall survival in AML patients. In the

Kaplan-Meier survival analyses (see *Online Supplementary Methods*), we found that high expression of DHODH was significantly correlated with poor prognosis in patients with cytogenetically normal AML based on data from a previous microarray study[20] (P=2.5 × 10^{-3}) (Figure 1A), suggesting a potential clinical role of human DHODH in AML.

Dihydroorotate dehydrogenase is required for maintenance of acute myeloid leukemia cancer cell malignancy

We next examined the levels of DHODH expression in a panel of human cancer cell lines including AML. We found that DHODH expression was higher in AML than in other cancer cell lines (Figure 1B), consistent with the results of bioinformatics analysis (*Online Supplementary Figure S1*) using large-scale cancer cell lines from the Cancer Cell Line Encyclopedia database.[21] We next silenced DHODH completely by a CRISPR/Cas9 knockout system in both HL60 and THP-1 cells. A substantial knockout of the DHODH protein was observed in the knockout groups compared with the control group (Figure 1C and *Online Supplementary Figure S2A*). Notably, DHODH knockout impaired the growth of HL60 and THP-1 cells (Figure 1D and *Online Supplementary Figure S2B*). Knockout of DHODH caused an increase of HL60 cell apoptosis from 1.34±0.21% to 23.47±1.23% (sgRNA1) or 26.18±0.84% (sgRNA2), compared to the control (Figure 1E). Similar cell apoptosis induction was observed in THP-1 (from 4.72±0.41% to 19.93±1.74% (sgRNA1) or 21.79±1.32% (sgRNA2) (*Online Supplementary Figure S2C*).

Western blot analysis further showed significant upregulation of three apoptosis-related markers (cleaved PARP, cleaved caspase-3 and cleaved caspase-9) in HL60 and THP-1 cells, thus revealing an apoptotic mechanism (Figure 1F and *Online Supplementary Figure S2D*).

We observed that DHODH-knockout significantly increased the expression of CD11b and CD14 (differentiation markers of myeloid cells), whereas it had no effect on CD33 and CD34, in either HL60 (Figure 1G) or THP-1 cells (*Online Supplementary Figure S2E*), suggesting that DHODH-knockout induces myeloid differentiation of AML cells. The best-known MYC protein family member, MYC, a crucial myeloid cell differentiation modulator, is frequently overexpressed in AML.[22] Bioinformatics analysis revealed that DHODH is highly co-expressed with MYC in AML patients (*Online Supplementary Figure S3*) according to RNA-sequencing data from The Cancer Genome Atlas.[23] We found that DHODH knockout significantly reduced the expression of MYC protein in HL60 and THP-1 cells (Figure 1H and *Online Supplementary Figure S2F*). p21 is transcriptionally suppressed by MYC in cancer cells.[24] Notably, MYC loss induced by DHODH inhibition accompanied an elevation of protein expression of p21 in HL60 and THP-1 (Figure 1H and *Online Supplementary Figure S2F*). Taken together, abrogation of DHODH activity in AML markedly alleviated malignant characteristics, indicating that DHODH is a potential therapeutic target in AML.

Isobavachalcone is a novel dihydroorotate dehydrogenase inhibitor

Through screening an in-house natural product library using the 2,6-dichloroindophenol assay (see *Online Supplementary Methods*), we found that, at the concentration of 10 μM, isobavachalcone, a chalcone derived from traditional Chinese medicine *Psoralea corylifolia*, showed the greatest inhibitory activity on recombinant human DHODH protein among 337 natural products (Figure 2A). The chemical structure of isobavachalcone is presented in Figure 2B. Specifically, isobavachalcone showed a half maximal inhibitory concentration (IC_{50}) value of 0.13 μM on DHODH, which is approximately 2-fold stronger than that of leflunomide, a Food and Drug Administration-approved DHODH inhibitor for the treatment of rheumatoid arthritis (Figure 2C).[25] To further examine the direct interaction between isobavachalcone and DHODH, we first performed a thermal shift assay, a commonly used assay to evaluate ligand-protein interaction.[26] Figure 2D reveals that isobavachalcone significantly stabilized DHODH protein with an over 14°C melting temperature (ΔTm) increase in the presence of a 10-fold molar excess of DHODH (Figure 2D), suggesting a direct interaction between isobavachalcone and DHODH. Furthermore, we observed a dose-dependent attenuation of signal in the Carr-Purcell-Meiboom-Gill nuclear magnetic resonance (NMR) spectra, confirming that DHODH influences the state of isobavachalcone (Figure 2E).[27] In addition, isobavachalcone bound to DHODH with a K_D value of 1.33 μM (Figure 2F), according to an isothermal titration calorimetry experiment, which is consistent with results of the thermal shift assay and NMR experiments.

We next performed kinetic analysis of isobavachalcone against human DHODH using a Lineweaver-Burk plot. We found that isobavachalcone is a competitive inhibitor against coenzyme Q_0 and uncompetitive for the substrate dihydroorotate (*Online Supplementary Figure S4*). Figure 2G reveals that isobavachalcone occupies the "ubiquinone channel", a well-known ligand binding pocket of DHODH (PDB ID: 4YLW),[28] as determined from molecular docking simulation (see *Online Supplementary Methods*). Specifically, several hydrophobic contacts are involved in the binding between isobavachalcone and amino acid residues of DHODH, including Tyr38, Gln47, Ala55, Ala59, Leu67, Phe98, Val143, Thr360 and Pro364. In brief, we identified a direct, high potential DHODH inhibitor, isobavachalcone, which could be a therapeutic agent for AML.

Isobavachalcone inhibits the proliferation of acute myeloid leukemia cells via inhibition of dihydroorotate dehydrogenase

We next investigated DHODH protein expression in four human AML cell lines: HL60, THP-1, U937 and MOLM-13. Among these four AML cell lines, HL60 has a high level of DHODH protein and is sensitive to isobavachalcone (*Online Supplementary Figure S5A,B*). Figure 3A,B shows that isobavachalcone significantly suppresses HL60 and THP-1 cell proliferation in a concentration-dependent and time-dependent manner. The cell-counting assay further revealed isobavachalcone concentration- and time-dependent suppression of HL60 cell growth (*Online Supplementary Figure S5C*). Importantly, Figure 3C illustrates that both recombinant DHODH and endogenous DHODH in HL60 cells bind directly bind to isobavachalcone-conjugated-Sepharose 4B beads, but not to Sepharose 4B beads alone. Isobavachalcone stabilized DHODH in HL60 cells in a cellular thermal shift assay (Figure 3D), suggesting that isobavachalcone inhibits DHODH directly in AML cells. Notably, knockout of

DHODH markedly reduced the sensitivity of HL60 cells to isobavachalcone (Figure 3E). Taken together, these findings indicate that isobavachalcone suppresses HL60 cell growth through direct inhibition of DHODH.

After treatment with increasing concentrations of isobavachalcone for 72 h, the percentage of apoptotic cells increased in a dose-dependent manner (Figure 3F,G). To further investigate the mechanisms underlying isobavachalcone-induced apoptosis in HL60 cells, we performed a western blot assay to detect the protein marker of apoptosis. Figure 3H reveals that several protein markers of apoptosis (cleaved caspase-9, cleaved caspase-3 and cleaved

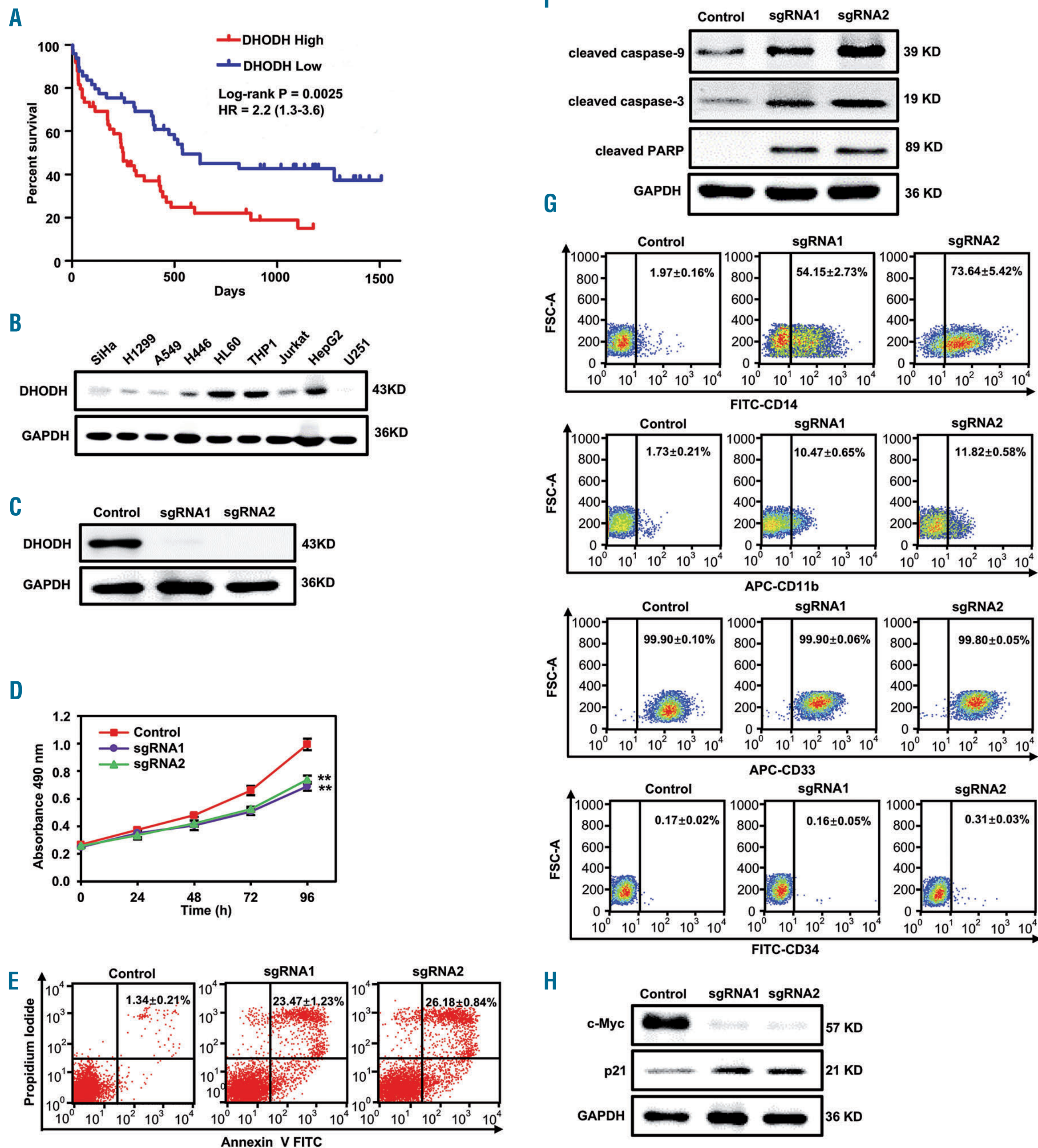

Figure 1. Dihydroorotate dehydrogenase is required for acute myeloid leukemia cells to maintain their malignant characteristics.. (A) Kaplan-Meier survival curves for AML patients divided by level of DHODH expression. The *P*-value of the Kaplan-Meier survival analysis was determined using a log-rank test (see the *Online Supplementary Methods*). (B) Western blot analysis of the expression levels of DHODH in different cancer types. SiHa: cervical carcinoma; H1299, A549 and H446: lung carcinoma; HL60 and THP1: AML; Jurkat: acute T-cell leukemia; HepG2: hepatic carcinoma; U251: glioma. (C) Knockout of DHODH in HL60 cells was analyzed by western blot. (D) Knockout of DHODH impaired the growth of HL60 cells. Cell viability was evaluated by MTS assay at 24 h intervals up to 96 h in three independent experiments. The graph represents the means ± SD. The Student *t*-test was performed, $^{**}P<0.01$. (E and F) DHODH knockout resulted in apoptosis of HL60 cells. Cell apoptosis was analyzed by flow cytometry and the expression levels of apoptosis-related proteins in HL60 cells was detected by western blot at 96 h after infection. (G) Flow cytometry demonstrated upregulation of cell surface markers CD14 and CD11b after knockout of DHODH in HL60 cells whereas there was no effect on CD33 and CD34 expression. The cells were measured at 96 h after infection. Data represent the mean ± SD of three independent experiments. (H) Knockout of DHODH resulted in reduced expression of MYC protein and upregulated expression of p21 in HL60 cells.

PARP) are increased after isobavachalcone treatment. Given that DHODH represented the rate-limiting step of *de novo* pyrimidine biosynthesis in the endogenous synthesis of uridine monophosphate, we wondered whether uridine could rescue isobavachalcone-induced apoptosis. Figure 3F-H shows that uridine alone did not affect cell apoptosis, whereas it reversed apoptosis induced by isobavachalcone treatment. Additionally, we examined the effect of isobavachalcone on cell morphology using fluorescence microscopy. Chromatin condensation was observed with Hochest 33258 staining after treatment with 30 μM isobavachalcone for 48 h (Figure 3I). Similar apoptotic events following isobavachalcone treatment were also observed in THP-1 cells (*Online Supplementary Figure S6*). It can be concluded that isobavachalcone induces apoptosis of HL60 cells by inhibiting DHODH activity.

Isobavachalcone triggers differentiation by inhibiting dihydroorotate dehydrogenase

We then investigated whether isobavachalcone triggers

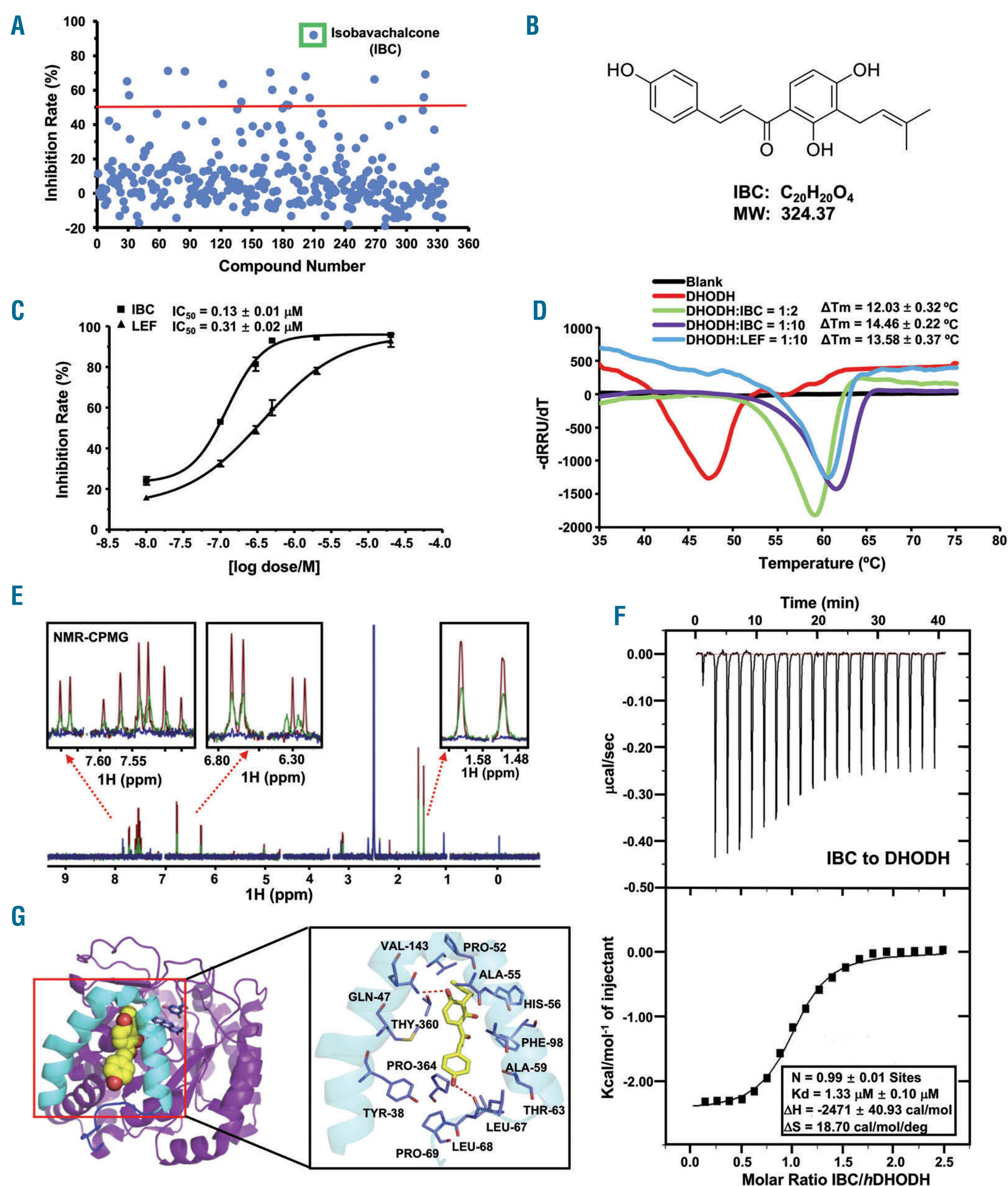

Figure 2. A natural product, isobavachalcone, is a newly identified direct dihydroorotate dehydrogenase inhibitor. (A) Graphical presentation of screening results of 337 compounds tested at a concentration of 10 μM in a DHODH enzymatic assay. Each dot represents one compound. (B) Chemical structure of isobavachalcone. (C) Dose-response curves of isobavachalcone and leflunomide in the DHODH enzymatic assay. (D) A thermofluor assay shows that isobavachalcone robustly stabilizes DHODH and produces a thermal shift over 14°C (ratio 1:10). (E) NMR measurement of direct binding between isobavachalcone and DHODH. Carr-Purcell-Meiboom-Gill NMR spectra for isobavachalcone (red), isobavachalcone in the presence of DHODH at 2.5 μM (green) and 5 μM (cyan). (F) Isothermal titration calorimetry of isobavachalcone binding to DHODH. Binding curves were fitted as a single binding event. (G) Computational docking analysis of the binding mode of isobavachalcone with DHODH. The structure is shown as a ribbon diagram and the isobavachalcone molecule (left) is presented as a sphere model based on PDB ID: 4YLW. The amino acid residues surrounding isobavachalcone (yellow sticks, right) are represented by slate sticks. Figure 1G was generated by PyMOL software (https://www.pymol.org/). IBC: isobavachalocone; LEF: leflunomide.

AML cell differentiation. We measured the level of expression four myeloid differentiation markers, CD14, CD11b, CD33 and CD34, by flow cytometry. Of note, we found that isobavachalcone increased the expression of CD14 and CD11b (Figure 4A,B), but had no effect on the expression of CD33 and CD34 (*Online Supplementary Figure S7A*) in HL60 cells. As DHODH is involved in the intracellular synthesis of uridine,[29] we observed that isobavachalcone treatment lead to the depletion of uridine in HL60 cells (*Online Supplementary Figure S8A*). The uridine rescue experiment demonstrates that the endogenous cellular pyrimidine deficiency induced by DHODH inhibition is crucial for the differentiation of AML cells (Figure 4A,B and *Online Supplementary Figure S7B*).

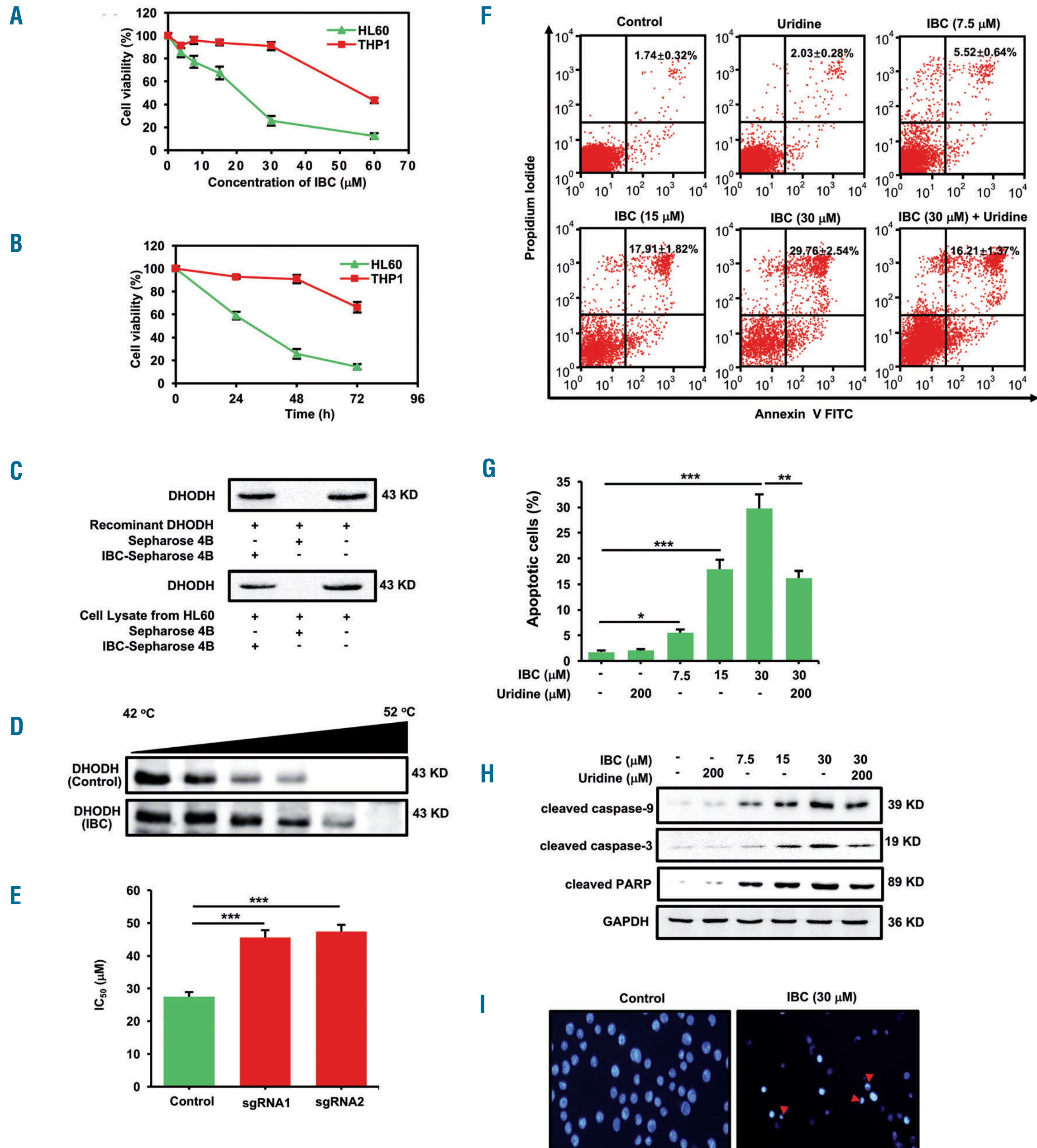

Figure 3. Isobavachalcone shows anti-proliferative activity against acute myeloid leukemia cells. (A) HL60 and THP1 cells were treated with increasing concentrations of isobavachalcone for 48 h, and cell viability was measured by MTS assay. (B) The time-response curve of 30 μM isobavachalcone on cell viability of HL60 and THP1 cells. (C) Recombinant DHODH protein or HL60 cell lysate was incubated with control or isobavachalcone-conjugated Sepharose 4B beads. Proteins bound to the beads were analyzed by western blot. (D) Cellular thermal shift assay shows that isobavachalcone stabilizes and targets DHODH in intact HL60 cells. Cells were incubated with isobavachalcone for 12 h and the assay was performed. (E) The IC_{50} value of isobavachalcone against DHODH-knockout HL60 cells. (F) HL60 cells were treated with isobavachalcone at the indicated concentrations for 72 h. Cell apoptosis was detected by flow cytometry using staining with annexin V, fluorescein isothiocyanate (FITC) and propidium iodine (PI). (G) The quantitative data of cell apoptosis in (F). (H) Changes in apoptosis-related proteins after treatment with isobavachalcone or uridine for 72 h. (I) Representative images of Hoechst 33258-stained cells were analyzed by fluorescence microscopy in HL60 cells treated with 30 μM isobavachalcone for 48 h. Red arrows indicate apoptotic cells. IBC: isobavachalocone.

Wright–Giemsa staining shows that isobavachalcone strikingly reduced the nuclear cytoplasmic ratio (N: C ratio) compared to the control (Figure 4C), suggesting monocytic differentiation. MYC is a main regulator of AML cell differentiation, and DHODH knockout reduces the protein level of MYC in HL60 cells (Figure 1H). As expected, after isobavachalcone treatment, there was a significant concentration-dependent loss of MYC expression accompanied by a significant up-regulation of p21 (Figure 4D). Furthermore, MYC protein level was down-

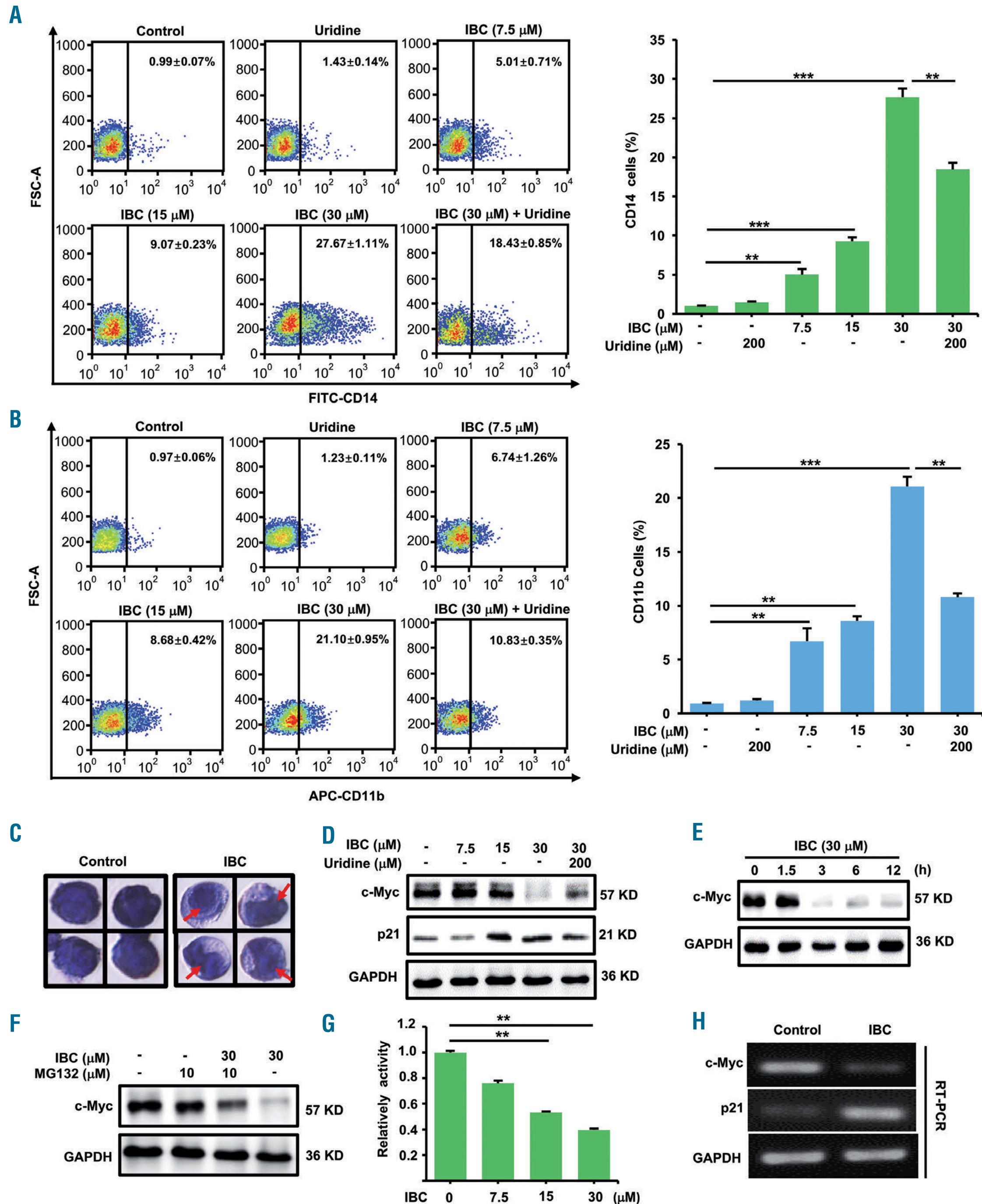

Figure 4. Isobavachalcone induces HL60 cell differentiation. (A) HL60 cells were treated with isobavachalcone for 72 h and CD14 expression was detected by flow cytometry analysis. Right: quantification data of CD14 expression in HL60 cells. (B) HL60 cells were treated with isobavachalcone for 72 h and CD11b expression was detected by flow cytometry analysis. Right: quantification data of CD11b expression in HL60 cells. (C) Morphological changes associated with differentiation of HL60 cells were evidenced by Wright-Giemsa staining in the presence of 30 μM isobavachalcone. (D) HL60 cells were incubated with different concentrations of isobavachalcone for 24 h. After incubation, western blot assay was performed to examine the expression levels of MYC and p21. (E) The expression of MYC was analyzed by western blot 1.5, 3, 6 and 12 h after treatment with 30 μM isobavachalcone. (F) HL60 cells were treated with 30 μM isobavachalcone for 3 h with or without 10 μM MG132. The levels of MYC expression were subsequently examined by western blot analysis. (G) 293T cells were transfected with the MYC-luc reporter plasmid together with pRSVluc plasmid (as an internal control) and incubated with different concentrations of isobavachalcone for 24 h. (H) HL60 cells were treated with 30 μM isobavachalcone for 24 h, then MYC and p21 gene levels in HL60 cells were examined by reverse transcriptase polymerase chain reaction. Data represent mean ± SD of three independent experiments. A Student *t*-test was performed, **P*<0.05, ***P*<0.01, ****P*<0.001. IBC: isobavachalocone.

regulated by isobavachalcone in a time-dependent manner (Figure 4E). MYC has been reported to be an unstable protein that is degraded rapidly via the proteasome pathway.[30] As shown in Figure 4F, isobavachalcone induced significant degradation of MYC, and treatment with MG132 (a proteasome inhibitor) blocked MYC deregulation. Moreover, extended isobavachalcone treatment (24 h) reduced *MYC* gene transcription in a *MYC* reporter assay (Figure 4G). Figure 4H further confirms down-regulation of *MYC* and *p21* gene expression following isobavachalcone treatment.

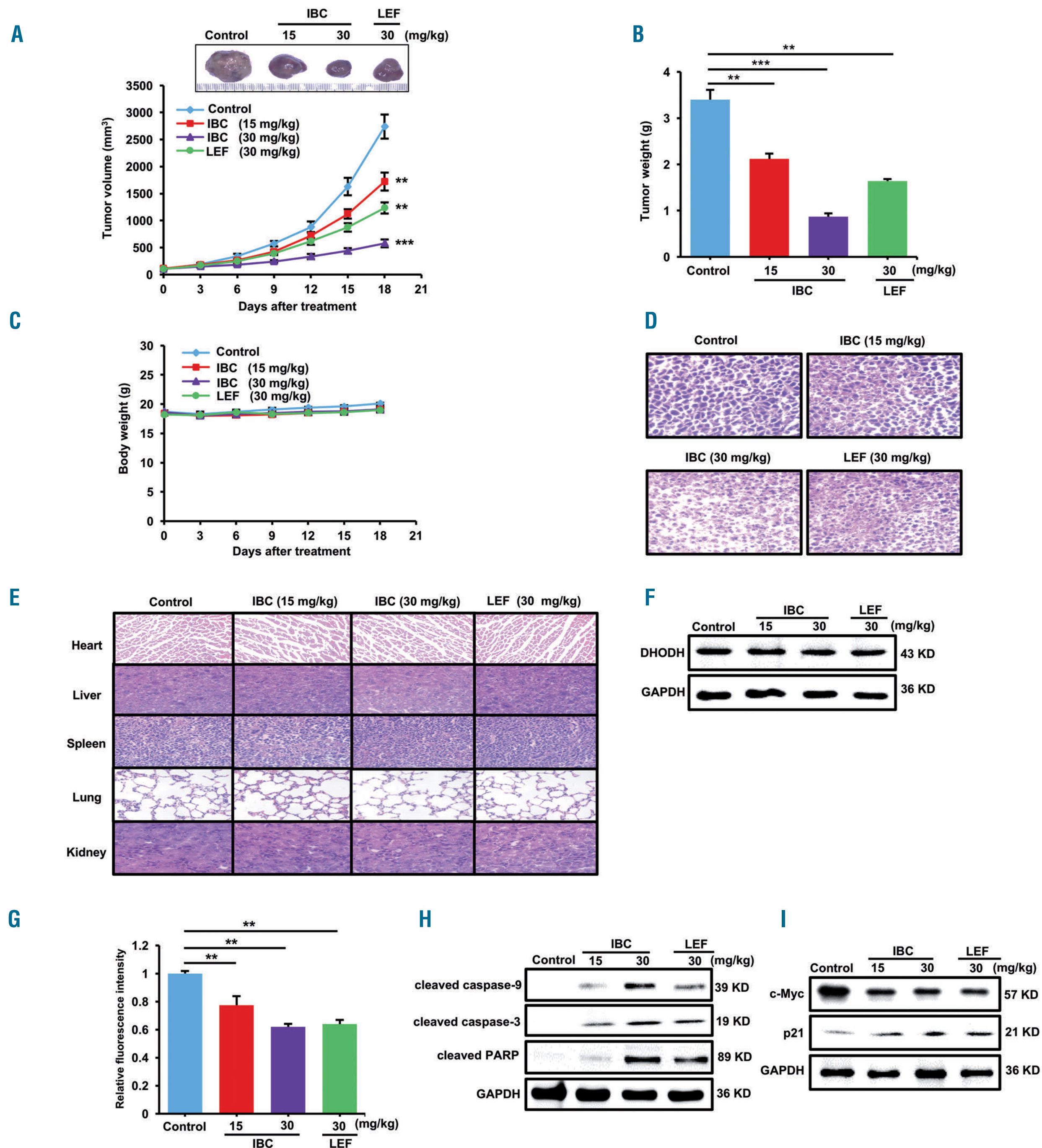

Figure 5. Isobavachalcone suppresses tumor growth in a subcutaneous HL60 xenograft mouse model. (A) Measurements of tumor volume in an HL60 xenograft mouse model treated with vehicle or the indicated dosages of compounds for 18 days. Changes in mean tumor sizes compared with the control group. Bars represent mean ± SD for eight animals in each group. (B) Effect of isobavachalcone on tumor weights. (C) The body weight of mice was measured every 3 days. (D) Histological morphology of tumor tissue, stained with hematoxylin and eosin, from the different groups. (E) Images of the major organ tissues, stained with hematoxylin and eosin, from the different groups of animals. (F) DHODH protein expression in tumors was examined at the time the animals were sacrificed. (G) The enzyme activity of DHODH in xenograft tumor tissues treated with vehicle, isobavachalcone or leflunomide was measured by fluorescence assay. (H) Western blot analysis of the changes of apoptosis-associated proteins at day 18 of HL60 xenograft tumors treated with vehicle, isobavachalcone or leflunomide. (I) Expression levels of MYC and p21 in tumors treated with vehicle, isobavachalcone, or leflunomide were estimated by western blot. Data represent means ± SD. A one-way ANOVA test was performed, ***P*<0.01 and ****P*<0.001. IBC: isobavachalocone; LEF: leflunomide.

Previous studies have shown that *O*-linked *N*-acetylglucosamine transferase (OGT) is directly involved in the regulation of MYC expression.[31,32] As expected, our results further showed that isobavachalcone treatment led to a significant reduction of OGT (*Online Supplementary Figure S8B*). Taken together, these findings indicate that the inhibition of DHODH by isobavachalcone induces MYC degradation-dependent differentiation of AML cells.

isobavachalcone exhibits antitumor efficacy by inhibiting dihydroorotate dehydrogenase activity *in vivo*

Figure 5A shows that isobavachalcone significantly suppressed tumor growth (37.81 ± 4.32% and 78.91 ± 9.73%, 15 and 30 mg/kg isobavachalcone, respectively), compared with the control group, in a subcutaneous HL60 xenograft mouse model. Similarly, the weight of tumors in the groups of animals treated with isobavachalcone was significantly reduced (Figure 5B) by 37.65 ± 3.74% and 74.41 ± 8.47%, respectively. No obvious body weight loss or deaths were observed in any of the groups of mice (Figure 5C), suggesting that isobavachalcone has a low toxicity *in vivo*. Hematoxylin and eosin staining analysis further supports the potent tumor suppression exerted by isobavachalcone on the HL60 xenograft model (Figure 5D) without significant damage to the main organs of the mice treated with this compound (Figure 5E). We then examined the expression level and activity of DHODH protein *in vivo.* Western

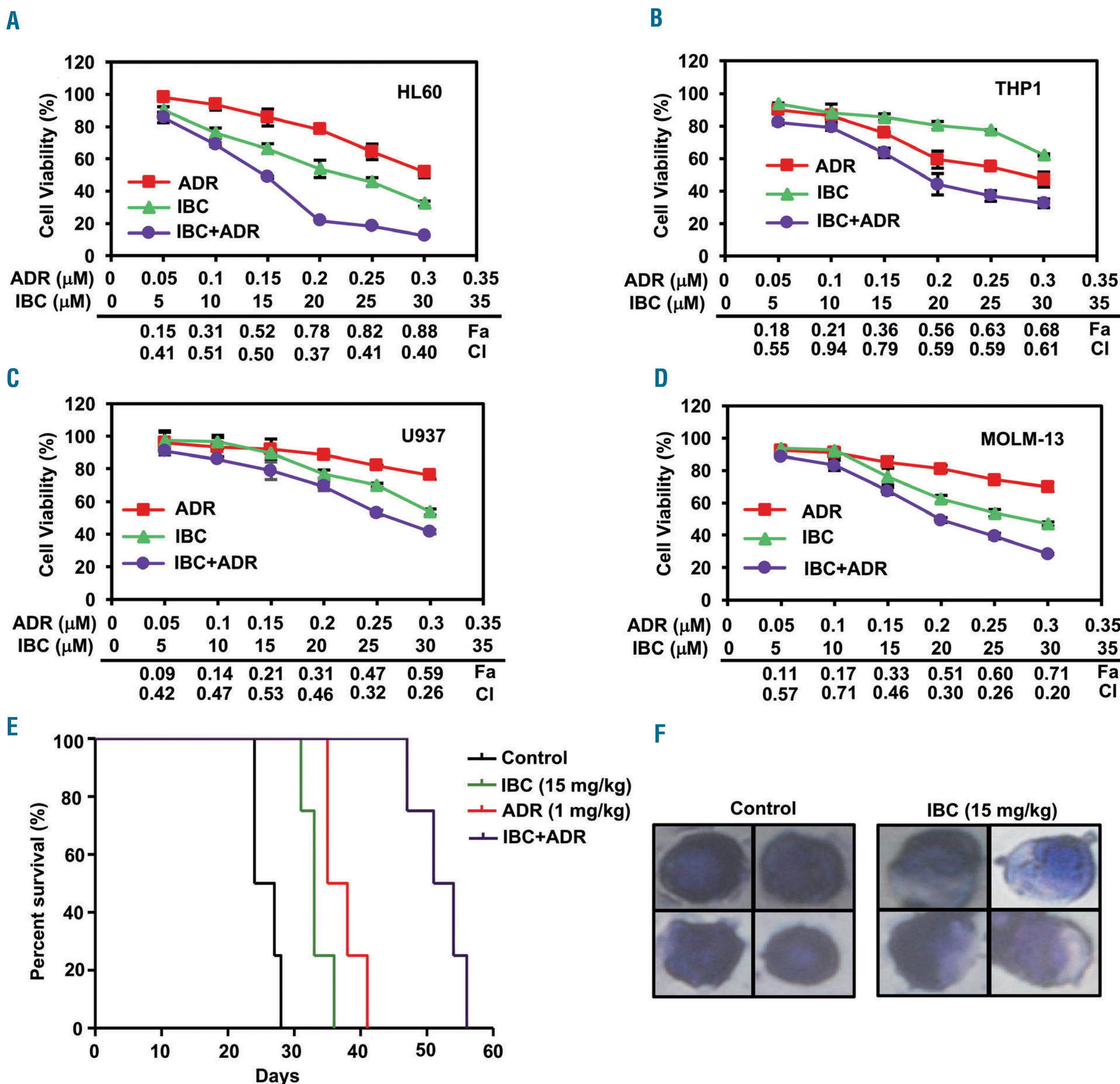

Figure 6. The combination of isobavachalcone and adriamycin shows synergistic antileukemic effects *in vitro* and *in vivo*. (A-D) Synergistic effects of the isobavachalcone and adriamycin combination on AML cells. AML cell lines (HL60, THP1, U937 and MOLM-13) were treated with several increasing concentrations of isobavachalcone and adriamycin alone or in combination for 48 h. The combination index (CI) calculation was performed using CalcuSyn software (Version 2.1; Biosoft). Drug combinations with a CI<1 are considered to be synergistic. (E) *Synergistic* effects of isobavachalcone and adriamycin combination therapy in an intravenous HL60 leukemia model. Mice with established tumors (4 per group) were divided into four groups and treated with vehicle, isobavachalcone, adriamycin or a combination of isobavachalcone and adriamycin. The *P* value was determined using the log-rank test, *P*=0.0003 for the survival analysis (E). (F) Leukemia cells isolated from the isobavachalcone-treated group exhibit morphological features of differentiation. ADR: adriamycin; IBC: isobavachalocone.

blot revealed that DHODH expression levels were not affected in isobavachalcone-treated tumors (Figure 5F). Interestingly, DHODH activity decreased in isobavachalcone-treated tumors compared with vehicle-treated tumors, as determined by a cellular DHODH enzyme assay based on the use of a 4-trifluoromethyl-benzamidoxime fluorogenic reagent (Figure 5G). Hence, we hypothesized that isobavachalcone suppresses tumor growth by inhibiting DHODH enzymatic activity *in vivo*. We checked how isobavachalcone affects the expression levels of three apoptosis-related markers (cleaved caspase-9, cleaved caspase-3, and cleaved PARP) and two differentiation-related proteins (MYC and p21) in xenograft tumors. As expected, cleaved caspase-9, cleaved caspase-3 and cleaved PARP and p21 levels were noticeably elevated in the isobavachalcone-treated groups, while MYC protein level decreased (Figure 5H), consistent with the *in vitro* analysis (Figures 3H and 4D). Notably, isobavachalcone shows greater efficacy than leflunomide (Figure 5A-H).

The combination of isobavachalcone and adriamycin shows synergistic antileukemic effects *in vitro* and *in vivo*

Adriamycin is a widely used chemotherapy drug for treatment of AML in the clinic. However, as monotherapy, its therapeutic efficacy is limited by problems such as acquired resistance.[9] We investigated whether isobavachalcone sensitizes AML to adriamycin. We first determined the cytotoxicity of adriamycin in four human AML cell lines: HL60, THP-1, U937 and MOLM-13 (*Online Supplementary Figure S9*). We then examined the efficacy of a combination of isobavachalcone and adriamycin across the four AML cell lines and found that the isobavachalcone and adriamycin combination led to a cooperative suppression of AML cell growth (Figure 6A-D). We further investigated the potency of combinational therapy using a disseminated HL60 model of AML. As shown in Figure 6E, mice treated with the isobavachalcone and adriamycin combination had a significantly longer survival compared with that of animals in the other groups. Of note, treatment with isobavachalcone led to AML differentiation *in vivo*, as evidenced by Wright–Giemsa staining (Figure 6F).

We also investigated the effect of isobavachalcone on adriamycin-resistant HL60/adriamycin cells *in vitro*. As expected, the HL60/adriamycin cell line showed greater resistance to adriamycin (IC_{50} = 38.77±0.81 μM), (*Online Supplementary Figure S10A*) compared to HL60 cells (IC_{50} = 0.36±0.05 μM), (*Online Supplementary Figure S9A*). We found that isobavachalcone markedly suppressed HL60/adriamycin cell growth in a concentration-dependent manner (*Online Supplementary Figure S10B*). *Online Supplementary Figure S10C* shows that the combination of isobavachalcone and adriamycin had an enhanced antiproliferative effect in HL60/adriamycin cells compared with the effect of isobavachalcone or adriamycin alone *in vitro*. The values of the combination index, which were all <0.8, suggest a synergistic antitumor effect between isobavachalcone and adriamycin.[33] Notably, the group co-administered isobavachalcone and adriamycin exhibited markedly coordinative anti-tumor activity compared with monotherapy and control groups ($P<0.001$, one-way ANOVA) (*Online Supplementary Figure S10D,E*). No obvious changes were observed in the animals' body weight, indicating that the combined therapy was well tolerated (*Online Supplementary Figure S10F*). In summary, isobavachalcone combined with adriamycin effectively suppresses the growth of adriamycin-resistant AML cells *in vitro* and *in vivo*, offering a potential drug combination strategy for AML therapy.

Discussion

Differentiation therapy is inspired by the observation that hormones and cytokines can induce differentiation *ex vivo*; it can, therefore, be a powerful way of irreversibly altering the phenotype of malignant cells.[34] The high cure rates of acute promyelocytic leukemia by a combination of retinoic acid and arsenic underscore the success of differentiation therapy.[34] However, approximately 90% of patients with AML other than acute promyelocytic leukemia do not benefit from the combination of retinoic and arsenic. New differentiation therapy strategies are urgently needed to improve the clinical outcome of these patients. In this study, we demonstrated that DHODH is a potent regulator of AML cell growth, apoptosis and differentiation. Specifically, genetic knockout or pharmacological inhibition of DHODH overcomes a differentiation blockade of myeloid cells by promoting MYC degradation. Via systematic screening of an in-house natural product library, we identified isobavachalcone as an effective, direct DHODH inhibitor. By targeting DHODH, isobavachalcone suppressed tumor growth, overcoming the differentiation blockade of AML *in vitro* and *in vivo*, without a significant toxic profile, thus making it a potential therapeutic agent for AML differentiation.

MYC is a pro-oncogenic transcription factor that contributes to tumorigenesis and tumor progression of human cancers including leukemia.[35,36] MYC levels are correlated with tumor cell progression and differentiation and myeloid cell differentiation is dependent on the suppression of this transcription factor.[37] An earlier study revealed that inhibition of DHODH abrogates transcriptional elongation of the *MYC* gene in melanoma.[38] The DHODH inhibitor leflunomide can reduce MYC expression and consequently reduce proliferation of human melanoma cells.[38] However, the ramifications of DHODH inhibition on MYC in AML are still unclear. In our experiments, the level of MYC expression was markedly lowered after the silencing of DHODH, either by knockout of DHODH or by the introduction of the DHODH inhibitor. Isobavachalcone induced MYC degradation in a proteasome-dependent manner. In addition, extended isobavachalcone treatment inhibited MYC transcriptional activity in a luciferase reporter assay. Sykes and colleagues reported that inhibition of DHODH overcame differentiation blockade in AML; however, the precise pathway affected by DHODH inhibition is still not understood.[14] We suggest that inhibition of DHODH through down-regulation of MYC induces AML cell differentiation.

It was previously reported that isobavachalcone, a naturally occurring chalcone, exhibited anticancer activity in several types of malignancies. For instance, isobavachalcone showed anti-cancer activities in a two-stage mouse skin cancer model.[39] Yang *et al.* reported that isobavachalcone impaired the growth and increased apoptosis of the ovarian carcinoma cell line OVCAR-8 and prostate cancer

cell line PC3.[40] Isobavachalcone has been reported to inhibit AKT1 kinase in a dose-dependent manner *in vitro* with an IC_{50} value of 32.90 μM.[39] However, the anti-cancer properties and mechanisms of isobavachalcone are not fully understood. As described in this paper, we identified isobavachalcone as a potent, direct human DHODH inhibitor, and systematically validated it by, for example, enzymatic and isothermal titration calorimetry assays, thermal shift, and NMR. To the best of our knowledge, this is the first report of isobavachalcone's potent differentiation-inducing activity in AML and anti-leukemic effect in mouse xenograft models. However, other potential off-target effects caused by isobavachalcone in AML cells remain to be determined in the future. Taken together, these results provide compelling evidence of isobavachalcone's potent anti-leukemia activity by interfering with the biosynthetic pathway of pyrimidine nucleotides through suppression of DHODH catalytic activity.

Several major breakthroughs have been made recently in the diagnosis and therapy of AML; however resistance is still a daunting barrier.[9,23] It was reported that known DHODH inhibitors, leflunomide and A771726 can increase the sensitivity of cells to adriamycin in triple-negative breast cancer.[41] However, the biological consequences and clinical benefits of these agents in AML remain unclear. In this study, we demonstrated that the combination of the DHODH inhibitor isobavachalcone and adriamycin effectively suppressed the growth of AML cells and prolonged survival in xenograft models of AML without obvious toxicity, offering a promising therapeutic strategy for AML. The detailed molecular mechanism of the synergistic effect of this combination of drugs remains to be studied further. For example, *PTEN* is a well-known tumor suppressor gene and loss of *PTEN* is associated with chemoresistance in multiple types of cancers.[42] Recently, Deepti *et al.* revealed that *PTEN*-deficient cancers are heavily dependent on an intact pathway for *de novo* pyrimidine synthesis, which makes such tumors vulnerable to DHODH inhibition.[43] Thus, dual targeting of DHODH and mTOR/AKT may offer a strategy for further overcoming chemoresistance in AML patients with *PTEN* alterations.

In summary, we demonstrated that isobavachalcone triggers apoptosis and differentiation of AML cells via pharmacological inhibition of human DHODH, laying the groundwork for future AML treatment strategies. Importantly, administration of isobavachalcone enhanced the anti-cancer efficiency of adriamycin in a xenograft model of human AML. The comprehensive preclinical findings presented here suggest that isobavachalcone is a potential therapeutic agent for AML with a novel molecular mechanism, and that further development of the drug for use in the clinic is warranted.

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