Natural HDAC-1/8 inhibitor Baicalein exerts therapeutic effect in CBF-AML

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Research

Abstract

Background: Although targeting HDACs may be an effective strategy for CBF-AML harboring t(8;21) or inv(16), HDAC inhibitors are reported to be limited by drug-resistant characteristic. Our purpose is to evaluate the anti-leukemia effects of Baicalein on CBF-AML and clarify its underlying mechanism.

Methods: Enzyme activity assay was used to measure the activity inhibition of HDACs. Rhodamine123 and RT-qPCR were employed to evaluate the distribution of drugs and the change of ABC transporter genes. CCK8, Annexin V/PI and FACS staining certified the growth and differentiation effects of Baicalein. Duolink and IP assay assessed the interaction between HDAC-1 and ubiquitin, HSP90 and AML1-ETO, Ac-p53 and CBFβ-MYH11. AML cells-bearing NOD/SCID mice models were used to evaluate the anti-leukemic efficiency and potential mechanism of Baicalein *in vivo*.

Results: Baicalein showed HDAC-1/8 inhibition to trigger growth suppression and differentiation induction of AML cells. Although the inhibitory action on HDAC-1 was mild, Baicalein could induce the degradation of HDAC-1 via ubiquitin proteasome pathway, thereby up-regulating the acetylation of Histone H3 without promoting ABC transporter genes expression. Meanwhile, Baicalein increased the acetylation of HSP90 and lessened its connection to AML1/ETO, consequently leading to degradation of AML1-ETO in t(8;21) AML. In inv(16) AML, Baicalein possessed the capacity of apoptosis induction accompanied with p53-mediated apoptosis genes expression. Moreover, Moreover, CBFβ-MYH11-bound p53 acetylation was restored via HDAC-8 inhibition induced by Baicalein contributing the diminishing of survival of $CD34^+$ inv(16) AML cells.

Conclusions: These findings improve the understanding of the epigenetic regulation of Baicalein, and warrant therapeutic potential of Baicalein for CBF-AML.

Background

Acute Myeloid Leukemia (AML) is an aggressive hematological neoplasm caused by abnormal hematopoietic progenitor cells[1]. Based on the specific cytogenetic abnormalities as well as the French-American-British (FAB) classification of the leukemic cells and immunophenotype, AML can be classified into M0-M7 subtypes[2]. Most of AML patients harbor nonrandom and somatically acquired chromosomal aberrations including but not limited to inversion, insertions, deletion, trisomy, monosomy and reciprocal cytogenetic translocation [3, 4]. The core binding factor AML (CBF-AML) consists of 15% of adult AML and 30% of childhood AML, which has a favorable prognosis but the 5-year survival rate remains low[5].

CBF-AML encodes two recurrent cytogenetic abnormalities referred to as t(8; 21) and inv (16). The t(8; 21) is a common translocation identified in 40%-50% of FAB-M2 subtype, and rare cases of M0, M1 and M4 subtypes [6, 7]. Meanwhile, the inv(16) occurs in 5% of AML cases [8]. The abnormality is highly correlated with the AML subtype FAB-M4 with dysplastic eosinophils in bone marrow ($M4E_0$) [9, 10]. The CBFs consists of one CBF-β subunit and three possible CBF-α subunits, which are a group of DNA-binding

transcription factors [11, 12]. However, the fusion of AML1, whose encoding protein is a subunit of CBF-α, and ETO generates a novel gene AML1-ETO, resulting in a lack of the carboxyl terminal transactivation domain of AML1, which suggests that the AML1-ETO disrupts hematopoiesis through a dominant-negative mechanism[13]. The ETO recruits Histone deacetylase (HDAC) and associates with nuclear receptor corepressor (N-CoR), and acts to repress the transcription of AML1 target genes [14, 15]. Evidence show that the degradation of the AML1-ETO fusion protein is a target of this type of AML, and AML-ETO is a client protein of HSP90 reducing the stability of AML1-ETO and causing its degradation[16].

In the other type of CBF-AML, the inv(16) results in the fusion of CBF-β with MYH11 gene. The two nonampliflying inv(16) cases form two chimeric genes, CBF/MYH11 and MYH11/CBF. However, only CBFβ/MYH11 contributes to the leukemogenesis, which encodes a CBFβ-MYH11 smooth muscle myosin heavy chain (SMMHC) protein. Similar to AML1-ETO, the CBFβ-SMMHC (CM) form co-repressor complexes, leading to recruitment of HDAC and silence target genes [17, 18].

Interfering with the function of pro-leukemic fusion proteins is an effective strategy for AML treatment. HDACs are critical epigenetic modulating-factors implicated in cancer, especially in causation and progression of CBF-AML [19, 20]. The two types of fusion proteins in CBF-AML are both capable of recruiting HDACs, thus resulting in repression of target genes. HDAC inhibitors influence genes involved in cell differentiation, proliferation and survival. The expression of HDAC-1 is negative correlated with the prognosis and a specific target for inhibiting cell proliferation and leading to terminal differentiation in AML [21, 22]. As a substrate of HDAC-1, HSP90 can be inhibited through acetylation on lysine residues by HDAC-1[23]. HDAC-8 is another class I HDAC which has been reported to be overexpressed in neuroblastoma, glioma, childhood acute lymphoblastic leukemia and T-cell lymphoma [24, 25, 26]. HDAC-8 has been shown to interact with the CM chimeric protein and to impair acetylation and inactivation of p53 which bound to CM, thus promoting CM-associated leukemia stem cell (LSC) transformation and maintenance[27, 28].

HDAC inhibitors are widely investigated in cancer, which show synergistic effect with certain anti-cancer drugs [29, 30]. Vorinostat (also referred to as SAHA) and Romidepsin, two HDAC inhibitors, were approved for treating refractory cutaneous T-cell lymphoma (CTCL) clinically [31]. Despite the promising anti-cancer activities of HDAC inhibitors, clinical trials with HDAC inhibitors in solid tumor have not met success. Upregulation of multidrug resistance 1 (MDR1) and its encoding protein p-gp are believed to be a typical side-effect of HDAC inhibitors. Treatment of HDAC inhibitors, such as Apocodin, increased rhodamine-123 efflux and modulated MDR1 expression in Hela cells[32]. VPA, a HDAC-1 inhibitor, was found to increase the expression of MDR1 in HepG2, SW620, and KG1a cells [33, 34]. Moreover, TSA, a pan-HDAC inhibitor, displays high expression of MDR1 [35]. Sodium butyrate (NAB),another pan-HDAC inhibitor, could induce cell differentiation and accompanied with the increase of MDR1 [36]. What's more, HDAC inhibitors induce the upregulation of MDR1, BCRP, MRP7, and MRP8[37, 38]. Thereby, the slow progress of HDAC

inhibitors in clinical research may be associated with the ATP-binding cassette transporters. It is urgent to find effective agents which target and inhibit HDACs without triggering the multidrug resistance.

Baicalein, a flavonoid extracted from the root of *Scutellaria baicalenesis*, has been reported to mediate growth inhibition of human leukemia cells (K562 and HL-60) [39, 40]. We found that Baicalein showed inhibitory effects on HDAC-1/8, with no effects on the expression of ABC transporter genes which can be induced by most HDAC inhibitors. Moreover, we investigated the anti-proliferation activity of Baicalein *in vivo* and *in vitro* and its effects on differentiation and apoptosis of AML cells, especially in CBF-AML. We investigated the functional contribution of HDAC-1/8 in anti-leukemia effects, especially in CBF-AML, and evaluated the efficacy of Baicalein in AML cells-bearing mouse models.

Materials And Methods

Compounds and reagents

For *in vitro* experiment, Baicalein ($C_{15}H_{10}O_5$, MW: 270.24) with the purity of 97% as determined by HPLC, was dissolved in dimethylsulfoxide (DMSO) as a stock solution at 0.1 M and stocked at -20 °C. The working solution was freshly diluted with basal medium to final concentration before experiment. The final DMSO concentration of DMSO did not exceed 0.1%. Cells treated with the highest concentration of DMSO was as control in corresponding experiments. *In vivo* study, Baicalein was prepared as intragastric administration (0.5% sodium carboxyl methyl cellulose) by Dr. Xue Ke from College of Pharmacy, China Pharmaceutical University.

Fluorescein isothiocyanate (FITC) anti-human CD14 and phycoerythrin (PE) anti-human CD11b antibodies were obtained from eBioscience (San Diego, CA, USA). VPA was purchased from The First Affiliated Hospital of Nanjing Medical University. NAB was purchased from Aladdin and mice were injected intraperitoneally. TSA and PCI-34051 were obtained from CSNpharm and Z-VAD-FMK was obtained from Ape x Bio.

Cell Culture

Human AML cell lines U937, THP-1, Kasumi-1, SKNO-1 and ME-1 were from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology. Primary leukemia cells which from AML patients (The First Affiliated Hospital of Nanjing Medical University, Nanjing, China) were collected using lymphocyte-monocyte separation medium (Jingmei, China). The protocol of collection of cells from patients complied with guidelines in the Declaration of Helsiniki and was approved by the First Affiliated Hospital of Nanjing Medical University Hospital's Institutional Review Board and the appropriate ethics committees. A signed informed consent was obtained from patient. Primary AML cells and cell lines were cultured in RPMI-1640 medium, supplemented with 10% FBS, 100 U/ml of benzyl penicillin and 100 μg/ml of streptomycin

in a humidified environment with 5% CO2 at 37 °C. All cells used were passaged in our laboratory for less than 3 months after resurrection.

Animal Models

Female NOD/SCID mice (6–9 weeks old) (Beijing HFK Bioscience Co., Ltd, Beijing, China) were sublethally irradiated (1.8 Gy), and were engrafted with human primary AML cells (1×10^7 cells per mouse) via tail vein in 24 h following the radiation treatment. Animals in the control group were injected with physiological saline to evaluate the effects of injection on survival. Seven days later, the mice were injected by intragastric with or without Baicalein (80 mg/kg) every other day for 4 weeks. Next, the animals were inspected daily for 4 weeks. Finally, peripheral blood, bone marrow and spleen cells were prepared for flow cytometry after labeling with huCD45, and the cell lysate for Western blot. The BM biopsy was used to perform immunofluorescent staining.

Animals were maintained in an air-conditioned and pathogen-free environment (23 ± 2 °C, 55 ± 5% humidity) under controlled lighting (12 h light/day) and supplied with standard laboratory food and water ad libitum throughout the experimental period. The animal study was carried out according to the regulations of the China Food and Drug Administration (CFDA) on Animal Care.

Target Engagement Analyses

For enzyme activity assay, the purified protein HDAC was incubated with its substrate which contained acetylated lysine side chains. The substrate of HDAC-1/2/3/8 was K379-382 (RHKK(Ac)AMC) residue of p53. Deacetylation of the substrate causes the substrate to react with the lysine developer, the fluorescence was positive correlation with the deacetylation of substrate. Fluorescent signals generated by different concentrations of Baicalein were analyzed using a multi-function microplate reader. The positive control was the HDAC inhibitor TSA.

For cellular thermal shift assay, experiments were performed as described previously {Rozbeh, 2014 #281}.

Sirna Transfection

HDAC-1 and HDAC-8 siRNA were synthesized by Shanghai GenePharma Co, Ltd. Transfection was performed using Lipofectamine 2000™ reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions provided by the vendor. The siRNA sense oligonucleotides for HDAC-1 was 5'-AUAAACGCAUUGCCUGUGAUCAAAGAAGAGGUCAAGUU-3', and the anti-sense was 5'-UGACCAACCAGAACACUAAGAACUCUUCUAACUUCAAA – 3'. The siRNA sense oligonucleotides for HDAC-8 was 5'- CAUCGAAGGUUAUGACUGUUGACUAUGCAGCAGCUAUA – 3', and the anti-sense was 5'-CUACGUGGAUUUGGAUCUAGAUGAGAAGUACUAUCACA – 3'.

Differentiation Assay

Cell differentiation was assessed by NBT reduction and Giemsa Staining as previously reported[36]. Fluorescence intensity of CD11b and CD14 was analyzed with a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) [41]. Data were based on the examination of 10,000 cells per sample selected randomly from 5 × 105 cells.

Western Blot Analysis

Western blot was performed with standard protocols[42]. AML1-ETO was detected with anti-ETO antibody (1:500; Santa Cruz, CA). CBFβ-MYH11 was detected with anti-CBFβ antibody (1:1000; Abclonal Technology, China). Antibody against HDAC-1, Histone H3, acetyl-Histone H3, acetyl-p53(K382) were obtained from Cell Signaling Technology (1:1000; Beverly, MA). C/EBPα, HSP90, p53 and β-actin were obtained from Santa cruz (1: 500, CA). HDAC-8 and pan-acetyl-lysine were obtained from Abclonal Technology (1:1000, China). IRDyeTM 800-conjugated secondary antibodies were obtained from Rockland (Philadelphia, PA).

Immunofluorescence

Immunofluorescence was performed as previously described [43]. HDAC-1 was obtained from Santa cruz (1:50, CA). Alexa Fluor 488 donkey anti-goat IgG (H + L) antibody was purchased from Life Technologies (Carlsbad, CA, USA).

Quantitative Real-time Rt-qpcr

RT-qPCR was performed according to the manufacturer's instructions[44]. The primer sequences were as follows:

GAPDH

Forward 5'-GCAGGGGGGGAGCCAAAAGGG-3'

Reverse 5'-TGCCAGCCCCAGCGTCAAAG-3'
DR5

Forward 5'-CTGTGCATTCGTCTCTCTTGG-3'

Reverse 5'-TGAGTCGTTTCCGTTTACCG-3'
PUMA

Forward 5'-CACCCCATCGCCTCCTTTCT-3'

Reverse 5'-GGAAGGGGCGCGGACTGTCG-3'

NOXA

Forward 5'-AGATGCCTGGGAAGAAG-3'

Reverse 5'-AGTCCCCTCATGCAAGT-3'

Bax

Forward 5'-TCAAGGCCCTGTGCACTAA-3'

Reverse 5'-TGAGGACTCCAGCCACAAA-3'

PIG8

Forward 5'-GAAGGATGTGGCGAAGGGA-3'

Reverse 5'-CCACAAGACCGTCTACCTGCA-3'

Bcl-xl

Forward 5'-GATGCAGGTATTGGTGAGTCGG-3'

Reverse 5'-ATCCACAAAAGTGTCCCAGCC-3'

MDR1

Forward 5'-CATTGGCGAGCCTGGTAG-3'

Reverse 5'-TCGTAGGAGTGTCCGTGGAT-3'

MRP8

Forward 5'-AAACTTCTCTGTGGGGGAGA-3'

Reverse 5'-GGGTGTCTGTCTCCATGTCA-3'

MRP7

Forward 5'-CATGCAAGCCACGCGGAACG-3'

Reverse 5'-AAGCTGGGCTGGTGGAGGGT-3'

Facs Analysis Of Whole Blood

FACS analysis of whole blood was performed as previously described[43]. PE and FITC anti-human CD45 antibodies were obtained from Miltenyi Biotec Inc. (Auburn, CA, USA).

Duolink Assay

Duolink assay was performed according to the manufacturer's instructions [45]. Duolink in Situ Detection Reagents Red and Duolink In Situ PLA Probe Anti-Mouse PLUS Duolink In Situ PLA Probe Anti-Rabbit MINUS were purchased from Sigma-Aldrich.

Immunoprecipitation

Cells were pretreated with or without Baicalein for 24 or 48 h. Cell lysate was incubated with appropriate concentration of antibody and 20 μL protein A/G-conjugated beads (Santa Cruz) at 4 °C overnight. After three washing with RIPA buffer (Thermo Fisher), samples were collected and re-suspended in 20 μL SDS-sample buffer (0.5 M Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% 2-mercaptoethanol and 4‰ bromophenol blue) and boiled for 10 min. Then the samples were subjected to western blot.

Statistical analysis

All data were expressed as mean ± S.D. The data shown were obtained from triplicate independent parallel experiments. Statistical analysis of multiple group comparisons was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni *post-hoc* test. Comparisons between two groups were analyzed using two-tailed Student's *t*-tests. A P-value < 0.05 was considered statistically significant.

Results

Baicalein inhibits HDAC-1/8 activity without affecting the expression of HDAC inhibitors-associated ABC transporter genes

Emerging data now implicate histone modification is considered for a new therapeutic strategy in cancer including AML[46]. Histone acetylation is mediated by histone acetyltransferases, while acetyl groups are removed by HDAC[47]. To investigate the effect of Baicalein on HDACs activity, we performed enzyme activity assay and the data showed that Baicalein inhibited HDAC-1 and HDAC-8 activity (Fig. 1A). Cellular thermal shift assay (CETSA) is used to study thermal stabilization of proteins upon ligand binding. We therefore applied this method to further confirm the interaction between Baicalein and HDAC-1/8 but not HDAC-2/3 (Fig. 1B).

Multiple studies indicated that the exposure of cancer cells to HDAC inhibitors could induce the activation of several drug resistance-associated ABC transporters, which in turn lead to a broad-spectrum of drug resistance effect in the treatment of cancer[37]. Therefore, in order to further investigate the effect of Baicalein on the drug resistance, we used Rhodamine123 to evaluate the distribution of drugs on AML cell lines and primary AML cells [48]. Compared with VPA, a HDAC-1 inhibitor, as well as TSA, a pan-HDAC inhibitor, Baicalein showed little influence on the transport of Rhodamine123 (Fig. 1C, Table 1, Supplementary Fig. 1A), revealing that traditional HDAC inhibitors decreased the intracellular accumulation of Rhodamine-123 while Baicalein hardly decreased its accumulation. MDR1 is a ATP-binding cassette ABCB1 and a prognostic factor of AML treatment failure[49]. Moreover, multidrug resistance of HDAC inhibitors is associated with BCRP, MPR7 and MRP8 [50, 51, 52]. The mRNA levels of ABC transporter genes MDR1, BCRP, MRP7, and MRP8 dramatically increased after exposure to either VPA or TSA for 24 h in AML cell lines and primary AML cells, especially in the relapsed AML patient samples (#1,#2 and #28). However, the mRNA levels of those genes only slightly changed after treatment of Baicalein for 96 h (Fig. 1D).

Baicalein inhibits cell growth and induces differentiation of AML cell lines and primary AML cells

Human AML cell lines including U937, THP-1 and CBF-AML including Kasumi-1, SKNO-1 with t(8;21), ME-1 with inv(16) and primary AML cells were cultured in the absence or presence of Baicalein at various concentrations. Results showed that treatment of Baicalein at concentration ranging from 0 to 256 μM induced significant inhibition of cell viability in those cells, and have shown its superior performance in CBF-AML by CCK8 assay (Fig. 2A-B; Supplementary Fig. 2A-C).

We then examined the effect of Baicalein on the differentiation progression in AML cells. To confirm the differentiation induction effect of Baicalein on AML cells, we examined the expression of CD11b, a marker of myeloid differentiation, and CD14, a marker of monocytic maturation by FACs. The percentage of CD11b and CD14 positive cells were both increased after Baicalein treatment for 96 h in non-inv(16) AML cell lines (Fig. 2C, D) and primary AML cells (Table 2; Fig. 2E). Notably, t(8;21) AML cells were consistent with the potent differentiation induction after Baicalein treatment. However, Baicalein has no influence on the differentiation of ME-1 cells (Data not shown). Giemsa staining showed that Baicalein induces cell differentiation, which was indicated by the reduction of cell nuclear/cytoplasmic ratio, and adherence to the bottom of the culture flasks in non-inv(16) AML cells (Supplemental Fig. 2D). Furthermore, the NBT-reduction activity of non-inv(16) AML cell lines and primary AML cells also dramatically increased after treatment with Baicalein in those cells (Supplemental Fig. 2E).The expression of C/EBPα-p42, a leucine-zipper transcription factor which is vital in the development of granulocyte and neutrophil development where it acts as a tumor suppressor in the hematopoietic system was up-regulated by Baicalein in AML cell lines and primary AML cells. Interestingly, the expression of C/EBPα-p30 was down-regulated in cell lines including THP-1, U937, and #1 and #39 primary leukemia

cell, however, it mildly decreased in t(8;21) AML, such as Kasumi-1 and SKNO-1 (Fig. 2F-H). These results suggested that Baicalein inhibits cell growth and induces differentiation accompanied with an increase of p42/p30 ratio in AML cell lines and primary AML cells.

Baicalein Promotes Proteasome-dependent Degradation Of Hdac-1 In Aml Cells

Pharmacologic inhibition of HDACs induces differentiation, proliferation inhibition and apoptosis of AML cells [53]. Baicalein mildly inhibits HDAC-1 activity, while obviously inhibits HDAC-1 expression in AML cells and primary AML cells (Fig. 3A-C). The acetylation of Histone H3 was upregulated by Baicalein in AML cell lines and primary AML cells (Fig. 3A-C). Notably, the high background expression of HDAC-1 was consistent with the potent growth inhibition and differentiation induction by Baicalein in AML cells and primary AML cells (Fig. 2C-E, Supplementary Fig. 3A; Table 2). Even primary leukemia AML cells with low background expression of HDAC-1, Baicalein inhibited the expression of HDAC-1 protein as well (Supplementary Fig. 3B).

To understand the mechanism of Baicalein-induced HDAC-1 reduction, we first determined whether Baicalein affects HDAC-1 at transcriptional level. Kasumi-1 was treated with cyclohexumide (CHX), a protein synthesis inhibitor. Results showed that CHX didn't affect Baicalein-induced HDAC-1 decrease (Fig. 3D-F). We further adopted Actinomycin D (5 μg/ml), a transcription inhibitor. Results suggested that the down-regulation of HDAC-1 was not due to transcription inhibition (Fig. 3D,F). Moreover,either NH_4Cl, a lysosome inhibitor, or z-VAD-FMK, a broad-spectrum caspase inhibitor, was respectively applied in co-treatment with Baicalein. Results showed that Baicalein-triggered degradation of HDAC-1 had nothing to do with lysosome- and caspase-dependent pathways (Fig. 3D-F). Finally, we found that the Baicalein-induced degradation of HDAC-1 was blocked by MG-132, a proteasome inhibitor, indicating that the degradation of HDAC-1 by Baicalein may be dependent on ubiquitin proteasome pathway (Fig. 3D-F). Results of western blot showed that HDAC-1 began to degrade after treated by Baicalein for 48 h (Fig. 3G). Furthermore, Baicalein increased the conjugation of HDAC-1 and ubiquitin in AML cells (Fig. 3H), which was further confirmed by Duolink assay (Fig. 3I). Thus, the degradation of HDAC-1 by Baicalein may be involved in the ubiquitylation-proteasome.

Baicalein induces the degradation of AML1-ETO via decreasing its interaction with HSP90

To investigate whether the differentiation induction of AML cells by Baicalein is dependent on HDAC-1 expression, Kasumi-1 cells were transfected with HDAC-1 small interfering RNA (siRNA). The efficacy of transfection was monitored by using western blotting (Supplementary Fig. 4A).Cell differentiation analyses were subsequently performed by using FACS assay. Notably, HDAC-1 knockdown showed a certain degree of cell differentiation, but there was no significant enhance of in the degree of

differentiation after treatment of Baicalein, suggesting that the induction of differentiation of AML cells by Baicalein may depend on the inhibitory effect of HDAC-1 (Fig. 4A, B).

The molecular chaperone protein HSP90 stabilizes client oncogenic proteins like AML1-ETO which recruits transcription repression complex including HDAC-1 to repress AML1-regulated genes [54]. Baicalein inhibited the expression of AML1-ETO in Kasumi-1 and primary AML cells with t(8;21) in time- and dose-dependent manner (Fig. 4C, D). We further adopted Actinomycin D (5 µg/mL), a transcription inhibitor, suggesting that the change of AML-ETO protein was not affected by transcription (Supplementary Fig. 4B, C). Next, CHX was applied to examine the influence of Baicalein on protein synthesis of AML1-ETO and result suggested that blocking of protein synthesis did not affect Baicalein-induced reduction of AML1-ETO (Supplementary Fig. 4B, C). Further results showed that the protein interaction between AML1-ETO and HSP90 was interrupted by Baicalein, which might be caused by acetylation on lysine residues of HSP90 (Fig. 4E-G). Taken together, our results suggested that Baicalein induced the degradation of AML1-ETO via increasing HSP90 acetylation so as to interrupt their interaction.

Baicalein restores p53 acetylation and diminishes survival of CD34+

cells in inv(16) AML

We have so far confirmed the effect of Baicalein on HDAC-8 inhibition, and have shown its superior performance in inv(16) AML by CCK8 assay (Fig. 2A). Further data showed that Baicalein dramatically induces apoptosis in ME-1 cells and primary inv(16) AML cells (Fig. 5A, Supplementary Fig. 5A). Meanwhile, we used HDAC-8 inhibitor PCI-34051 to detect apoptotic induction in ME-1 cells. Results showed that the apoptosis could be induced by PCI-34051, but the efficacy is not as good as Baicalein (Fig. 5B). We speculated HDAC-8 inhibitor could induce expression of resistance-related genes, we next detected the expression of ABC transporter genes induced by PCI-34051 and Baicalein in ME-1 cells. We found that PCI-34051 significantly up-regulated mRNA expression of BCRP, MPR7 and MRP8 compared to Baicalein (Supplementary Fig. 5B). We also knocked down the expression of HDAC-8 to verify the pharmacodynamic mechanism of Baicalein on inv(16) AML. The efficacy of transfection monitored using western blotting (Supplementary Fig. 5C). The results showed that knockdown of HDAC-8 in ME-1 cells could induce apoptosis to a certain degree. However, after transfected with HDAC-8 siRNA, Baicalein does not enhance apoptotic effects of siRNA to HDAC-8 (Fig. 5C). We also used HDAC-1 inhibitor VPA to detect the apoptotic efficacy mediated by HDAC-1 inhibition in ME-1 cells, results showed that VPA hardly induce apoptosis of ME-1 cells (Supplementary Fig. 5D).

Recently research showed that CM fusion protein binds to p53 and HDAC-8 mediates CM-induced deacetylation of p53 in inv(16) AML {Qi, 2015 #8}. IP assay and Duolink assay were then performed, which showed that Baicalein increased the acetylation of CM-bound p53 without affecting p53 expression (Fig. 5D,E). The mRNA level of p53 downstream genes were also dramatically increased by

Baicalein in ME-1 and inv(16) positive sample #42 (Fig. 5I). However, Western bolt assay showed that the acetylation of p53 hardly changed after VPA treatment in ME-1 cells (Supplementary Fig. 5E). Inhibition of HDAC-8 selectively induces apoptosis of human inv(16) AML stem and progenitor cells{Qi, 2015 #280}. We isolated CD34$^+$ hematopoietic progenitor cells from inv(16) primary AML cells by magnetic activated cell sorting (MACS) (Fig. 5F). Notably, Baicalein induced a remarkable increase of apoptosis proportion in CD34$^+$ cells which was modulated by CM-p53-HDAC-8 complex (Fig. 5G, H). Overall, the results suggest that Baicalein modulates the CM-p53-HDAC-8 complex via inhibition of HDAC-8, resulting in an ascendant performance of Baicalein on inv(16) AML.

Baicalein Shows Anti-leukemic Activity In Aml Cells-bearing Nod/scid Mice

To further investigate the anti-leukemia activity of Baicalein *in vivo*, Kasumi-1 and ME-1 cells were injected in NOD/SCID mice via tail vein (Fig. 6A). After engrafted for 7 days, the mice were randomly assigned. We selected VPA (200 mg/kg) which showed better inhibition at HDAC-1 in t(8; 21) AML. Meanwhile, another pan-HDAC inhibitor NAB (200 mg/kg) was chosen as a positive control in inv(16) AML group because of its dominant position on the inhibition of HDAC-8. In Kasumi-1 cells-bearing NOD/SCID mice, we found that both of HDAC-1 and AML1-ETO expression significantly decreased in the cells come from bone marrow of Baicalein-treated group but not in the VPA-treated group (Fig. 6B). In ME-1 cells-bearing NOD/SCID mice, the acetylation of p53 markedly increased in the cells come from bone marrow of Baicalein-treated group but not in NAB-treated group (Fig. 6C). Additionally, Baicalein significantly reduced the amount of huCD45$^+$ cells, a marker of human leukocyte, in spleen and bone marrow of AML cell lines-bearing mice, while either VPA or NAB showed mildly decrease of the huCD45$^+$ cells (Fig. 6D, E). Further results showed that administration of either Baicalein or VPA/NAB dramatically prolonged survival compared to control group while Baicalein showed a better survival prolonging capacity (Fig. 6F, Supplementary Table 3).

To assess the *in vivo* anti-leukemia effect of Baicalein in human primary AML cells, we established two primary AML cells-bearing mice models engrafted by sample #29 with t(8; 21) and #39, a non-CBF-AML sample respectively. After administration of Baicalein (80 mg/kg) and VPA (200 mg/kg) every two days for 4 weeks, the survival was recorded. Baicalein showed a superior status of the median survival duration in sample #29 than in sample #39 (Fig. 6G, Supplementary Table 3). Meanwhile, after administration for a week, we chose mouse randomly in each group of sample #29-bearing mouse and killed them. We found that splenomegaly was obviously relieved after Baicalein treatment (Fig. 6H). Engrafted leukemia cells were obviously infiltrated into bone marrow of #29 sample-bearing mice. Notably, huCD45$^+$ sporadically distributed in the bone marrow of mice treated with Baicalein or VPA. In addition, either Baicalein or VPA treatment decreased the expression of HDAC-1 in huCD45$^+$ leukemia cells in bone marrow of #29 primary AML cells-bearing mice (Fig. 6J). Furthermore, the proportion of

huCD45^{+} cells in blood dramatically decreased in both Baicalein and VPA-treated groups in #29 sample-bearing mice (Fig. 6I).

Discussion

HDACs inhibition has been proved to show therapeutic effect through affecting proliferation, differentiation, and survival of AML cells. In the present study, we found that Baicalein mildly inhibited enzyme activities of HDAC-1/8 and induced degradation of HDAC-1 (Supplementary Fig. 6). Here, we found that Baicalein inhibited proliferation and induced differentiation of AML cell lines and primary AML cells derived from a cohort of AML patients identified as different FAB subtypes. In Fig. 1, The IC_{50} of non-inv(16) cell lines ranged from 15 μM to 65 μM after Baicalein treatment for 96 h. However, the IC_{50} of inv(16) cell line ME-1 was 4.825 μM. In addition, the apoptotic effect induced by Baicalein in ME-1 cells were much more intense and earlier than other AML cell lines, suggesting different mechanisms induced by Baicalein in inv(16) ME-1 and other AML cell lines. In non-inv(16) cell lines and primary AML cells, Baicalein showed differentiation induction to a more or less degree. Notably, Kasumi-1 and SKNO-1, two t(8; 21) AML cell lines, were more sensitive to Baicalein and the differentiation effect was much more outstanding than other cell lines.

In t(8;21) AML cells, HDAC-1 is involved in pathogenesis mechanism induced by fusion protein AML1-ETO produced by t(8; 21) [22, 55, 56]. In Fig. 3, we observed HDAC-1 degradation and C/EBPα up-regulation in AML cells after treatment of Baicalein for 96 h when differentiation could be detected. Besides, during the treatment of Baicalein, we also found the AML1-ETO degradation which was associated with the impairment of HSP90 chaperone function. Strategies to avoid resistance of HDAC inhibitors include employing combination therapies simultaneously targeting HDACs and HSP90 [57]. HSP90 is a molecular chaperone to stabilize longevity protein, and AML1-ETO is one of client proteins of HSP90. Furthermore, HDAC-1 has been reported to promote the deacetylation of HSP90 in the nucleus of human breast cancer cells [58]. Moreover, HDAC-1, HDAC6 and HDAC-10 have been shown to regulate HSP90 chaperoning VEGF receptor proteins [58, 59]. Baicalein is a natural product with broad spectrum of pharmacological functions and low toxicity. After treatment of Baicalein, the acetylation of HSP90 increased and the interaction of HSP90 and AML1-ETO could be reduced. Results of Immunoprecipitation and Duolink assay both proved that the binding of AML1-ETO and HSP90 was interrupted by Baicalein, which could be the key cause of degradation of AML1-ETO.

As we know, LSCs contribute to the leukemogenesis, and maintaining the relapse of AML[60]. Recent research has shown that CM fusion protein disrupted p53 activity through aberrant post-translational modification mediated by HDAC-8, thus promoting CM-associated LSCs transformation and maintenance [27]. In our study, we found that Baicalein showed better inhibitory effects in inv(16) AML cells and induced the apoptosis of ME-1 and primary AML cells with inv(16). In inv(16) AML, CM fusion protein recruits HDAC-8 and p53 as a protein complex in which p53 acetylation is impaired by HDAC-8 [27]. Therefore, we next explored the influence of Baicalein on CM-recruited p53. Results showed that Baicalein increased the

acetylation of CM-bound p53, while the p53 level was slightly changed. Results of RT-PCR assay showed that downstream genes of p53 were activated, which was consistent with increased acetylation of p53. Subsequently, we investigated the effect of Baicalein on CD34$^+$ inv(16) AML cells. Results showed that Baicalein induced a remarkable increase of apoptosis in the quiescent AML CD34$^+$ population. Taken together, Baicalein restored p53 acetylation in inv(16) AML cells and diminished survival of AML CD34$^+$ cells.

C/EBPα is a vital transcription factor in control of lineage-specific gene expression in hematopoiesis which acts as a tumor suppressor in a number of malignancies [61]. In several AML subtypes, C/EBPα expression is downregulated, resulting in a blockade of monocytic or granulocytic differentiation [62, 63]. mRNA of C/EBPα can be translated from two different AUG codes, giving rise to two distinct isoforms, p42 and p30 [64]. The p30 informs lacks the transactivation domain 1, which is required for C/EBPα to interact with TATA box-binding proteins and other transcription initiation factors. This truncated p30 isoform is known to inhibit p42 C/EBPα-mediated transactivation of target genes [65, 66]. The ratio of p42 to p30 reflects the differentiation level of AML cells, and differentiation inductors, such as ATRA, have been reported to increase the proportion of p42/p30 in THP-1, U937 and COCL-48[67, 68]. Notably, in Baicalein-induced differentiation effects in t(8; 21) AML cells, the level of p42 increased when p30 mildly decreased, which was different from a dramatic decrease of p30 in non-CBF AML cell lines including THP-1 and U937. As for the variance of C/EBPα regulation by Baicalein, we speculated that Baicalein exerted up-regulation of either p42 or p30 via the degradation effect of AML1-ETO fusion protein in AML with t(8; 21), but the influence by Baicalein in modulating p42/p30 ratio made the expression of p30 finally showed a slight change. In non-CBF-AML cells, Baicalein-induced differentiation may be more likely to associated with regulation on p42/p30 ratio, which needs to be further studied.

HDACs inhibitors are generally capable of causing the upregulation of MDR1, then inducing a broad and pleiotropic drug resistance of AML cells by regulating multiple ABC transporter genes, which is associated to poor prognosis [69]. Baicalein restored the acetylation of Histone via HDAC inhibition, though it didn't affect the expression of HDAC inhibition-associated ABC transporter genes. Recent research showed that Baicalein can inhibit TNF-α-induced NF-κB activation and expression of NF-κB-regulated target gene. Meanwhile, inhibition on NF-κB activity significantly decreased MDR1 gene expression and drug resistance in HL-60 cells, suggesting that Baicalein might be capable of suppressing MDR1 gene expression via inhibiting NF-κB signaling[70, 71]. In addition, previous studies have shown an inhibitory effect of Baicalein on p-gp expression and reversal of multidrug resistance *in vivo* and *in vitro* [36, 72, 73]. Above all, the activities of Baicalein in suppressing MDR1 gene and inhibiting p-gp might be the cause of remaining unchanged HDACs-inhibition-associated ABC transporter genes.

Even though HDACs inhibition strategy has been known as a promising approach for the treatment of cancers, the clinical efficacy of HDAC inhibitors was limited by board ABC-associated drug resistance in several cancers [36]. Our study suggests that Baicalein has HDAC-1/8 inhibition effects and exerts outstanding anti-leukemia effects in CBF-AML, while no effects on the expression of HDAC inhibitors-

associated ABC transporter genes could be observed. In CBF-AML, Baicalein showed a growth inhibition and differentiation induction in t(8; 21) AML cells and apoptosis effects in inv(16) AML cells, respectively. HDAC-1 inhibition by Baicalein caused AML1-ETO destabilization and expression of repressed genes which were associated to differentiation of t(8; 21) AML cells. Targeting HDAC-8 resulted in an improvement of p53 acetylation and apoptosis of CD34$^+$ cells with inv(16). This study demonstrates that Baicalein has a preferential inhibitory effect in CBF-AML cells, suggesting the potential of Baicalein in developing into a novel agent for the treatment of AML.

Conclusion

These findings improve the understanding of the epigenetic regulation of Baicalein, showed that Baicalein inhibited the activity of HDAC-1 and HDAC-8 without promoting ABC transporter genes expression and warrant therapeutic potential of Baicalein for CBF-AML.

Abbreviations

HDAC, Histone deacetylase; AML, acute myeloid leukemia; CBF, core binding factor; HSP90, core binding factor; FAB, French-American-British; N-CoR, nuclear receptor corepressor; SMMHC, smooth muscle myosin heavy chain; CM, CBFβ-SMMHC; LSC, leukemia stem cell; CTCL, cutaneous T-cell lymphoma; MDR1, multidrug resistance 1; NAB, Sodium butyrate; DMSO, dimethylsulfoxide; FITC, Fluorescein isothiocyanate; PE, phycoerythrin; CHX, cyclohexumide.

Declarations

Ethics approval and consent to participate

The animal study was carried out according to the regulations of the China Food and Drug Administration (CFDA) on Animal Care. All patients' samples were collected after informed consent in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of China Pharmaceutical University.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that no competing interest exists

Authors' contributions

Contribution: X.Y. designed and performed research, analyzed data, and wrote the paper; H.L. and X.L. performed research and analyzed data; P.H. and R.T. performed research; Y.Q. and X.W. collected data and performed statistical analysis; M.Z. and H.W. and Z.W. collected and analyzed data; J.X. provided the blood samples; and Q.G. and H.H. conceptualized the project and directed the experimental design and data analysis.

Authors' information (optional)

Not applicable

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Tables

Table 1

Clinical data for patient samples with AML

Patient NO.	Source	FAB	PB blast %	BM blast %	WBC	Cytogenetics	Status
#1	PB	M1	87.2	94		CEBPA/TET2	Relapsed
#2	PB	M1	98	98	369.3	OD	Relapsed
#3	PB	M1				OD	New
#4	PB	M1		95	190.02	DNTM3A/FLT3-ITD	New
#5	PB	M1		98	128.3	NPM1/WT1	New
#6	PB	M1		93	94.8	OD	New
#7	PB	M1	90.8	92	102.52	OD	New
#8	PB	M2b	55	80.96	8.5	AML1-ETO/WT1	New
#9	PB	M2b	2.79	35.5		AML1-ETO/WT1	New
#10	PB	M2b		7	7.2	CEBPA/AML1-ETO	New
#11	PB	M2b			32.5	AML1-ETO	New
#12	PB	M2b			89.07	TET2/WT1/AML1-ETO	New
#13	PB	M2b	44	<20	1.8	AML1-ETO	New
#14	PB	M2b		19	15.7	AML1-ETO	New
#15	PB	M2b			8.3	AML1-ETO/c-kit	Relapsed
#16	PB	M2a	94		111.4	OD	New
#17	PB	M2a	68	64	180.3	FLT3-ITD	New
#18	PB	M2a	21	82		DNTM3A/CEBPA	New
#19	PB	M2a		69	19.8	WT1/HOX11	Relapsed
#20	PB	M2a		94.83	43.84	OD	New
#21	PB	M2a	57.5	28	14.1	WT1/EVI1/DNTM3A	New
#22	PB	M2a		42.5	4.9	CEBPA	New
#23	PB	M2a			30.3	AML1-ETO	New
#24	PB	M2a	95	86.5	295.9	OD	New
#25	PB	M2a	29	49.26	2.5	WT1/CEBPA	New
#26	PB	M2a	25.5	30.88	1.8	OD	New
#27	PB	M2a		97	72.3	OD	New
#28	PB	M2	92	90	242.24	PHF6/ASXL1/PH6	Relapsed
#29	PB	M2			192.96	AML1-ETO	New
#30	PB	M2	23	19.68	2.67	WT1/HOX11	New
#31	PB	M2	22.8	46	28.8	TET2/CEBPA	New
#32	PB	M2	11		10.8	OD	New
#33	PB	M2				OD	New
#34	PB	M2			13.7	OD	New
#35	PB	M4				OD	New
#36	PB	M4b	2	20.5	300.07	OD	New
#37	PB	M4b	81	20	170	FLT3-ITD/NPM1	Relapsed
#38	PB	M5			21.41	WNT1	Relapsed
#39	PB	M5		71		TET2/IDH2/NPM1/FLT3	New
#40	PB	M5b	50		24.7	CBFB-MYH11/WT1	New
#41	PB	M5b	12	27.5	2.9	WT1/TLS-ERG	New
#42	PB	M4	94	94.4		CBFB-MYH11	New
#43	PB	M4	39	43.2		OD	New

Abbreviations: BM, bone marrow; FAB, French–American–British; OD, outside diagnosis; PB, peripheral blood; WBC, white blood cells count

Table 2

Expression of CD11b/CD14 in primary AML cells

Patient NO.	CD11b positive cells (%)				CD14 positive cells (%)			
	Baicalein (µM)				Baicalein (µM)			
	0	7.5	15	30	0	7.5	15	30
#1	4.38	18.33	51.9	61.16	1.78	3.99	12.12	42.36
#2	16.1	48	13	31	16.2	47	14	31
#3	11.48	20.8	40.9	52.11	15.09	23.5	43.6	53.93
#4	0.41	5.84	66.13	76.16	0.48	4.8	19.66	13.44
#5	38.61	60.77	64.99	66.1	40.05	45.08	23.32	13.39
#6	26.56	38.09	53.12	45.98	12.79	20.44	21.21	32.61
#7	25.78	27.77	26.94	31.02	20.55	21.92	11.26	12.19
#8	8.14	6.41	16.57	55.87	3.88	2.62	5.04	29.5
#10	1.97	6.81	59.38	30.37	1.97	19.87	17.66	37.6
#11	28.57	58.12	59.62	63.43	5.14	9.9	51.43	58.6
#12	37.94	48.21	55.62	56.64	39.1	52.27	11.26	15.68
#13	4.6	7.47	16.61	21.31	1.1	3.72	6.98	7.94
#18	10.71	17.06	30.92	75.56	6.47	9.17	13.92	16.18
#19	1.5	33.83	36.28	43.23	2.87	46.75	21.32	19.96
#21	20.3	20.34	20.61	19.37	11.29	10.53	14.12	12.72
#22	10.7	24.07	39.91	80.02	7.92	13.74	24.14	25.67
#23	18.12	29.56	35.26	61.07	14.28	25.22	14.93	18.7
#24	8.46	25.73	22.71	20.67	6.97	13.67	13.09	14.6
#25	9.23	17.98	36.21	50.23	7.94	14.33	18.99	25.44
#27	9.17	9.36	11.4	44.48	6.14	60.6	6.53	38.48
#28	5.32	2.08	4.35	5.96	3.17	1.28	2.34	3.96
#29	21.2	28.28	19.44	36.27	6.49	7.03	7.64	13.67
#31	69.19	85.22	89.04	92.8	59.74	77.34	13.89	11.29
#32	16.52	25.9	34.52	50.67	2.47	8.45	17.85	34.23
#33	11.02	9.7	29.5	44.78	45.3	49.14	36.92	32.85
#34	18.03	3.61	5.47	18.2	1.37	0.8	5.01	10.84
#35	16.8	45.04	38.24	38.02	13.53	21.69	19.65	17.43
#36	28.75	31.82	52.37	65.63	22.29	25.21	10.37	13.96
#37	6.11	14.95	30.19	49.45	2.62	11.44	18.69	27.76
#38	27.32	26.16	33.66	39.45	24.71	24.09	29.46	34.49
#39	25.3	60.38	64.87	66.44	9.9	19.02	24.34	35.52
#40	6.53	55.32	54.38	61.34	9.42	18.2	20.1	23.2
#41	26.42	28.9	34.8	38.33	7.13	14.98	23.32	30.66
#42	9.2	13.2	12.3	11.4	4.5	2.5	4.09	4.05
#43	33.76	35.4	35.8	44.79	14.13	14.21	14.29	22.19

Table 3

Median Survival of AML cells-bearing NOD/SCID mice

Kasumi-1 cells-bearing NOD-SCID mice		ME-1 cells-bearing NOD-SCID mice	
	Median Survival (Days)		Median Survival (Days)
Control	15.5	Control	13.5
VPA	28.5	NAB	16
Baicalein	33.5	Baicalein	35
#29 sample-bearing NOD-SCID mice		#39 sample-bearing NOD-SCID mice	
	Median Survival (Days)		Median Survival (Days)
Control	14.5	Control	8.5
VPA	36.5	VPA	14.5
Baicalein	56.5	Baicalein	16

Figures
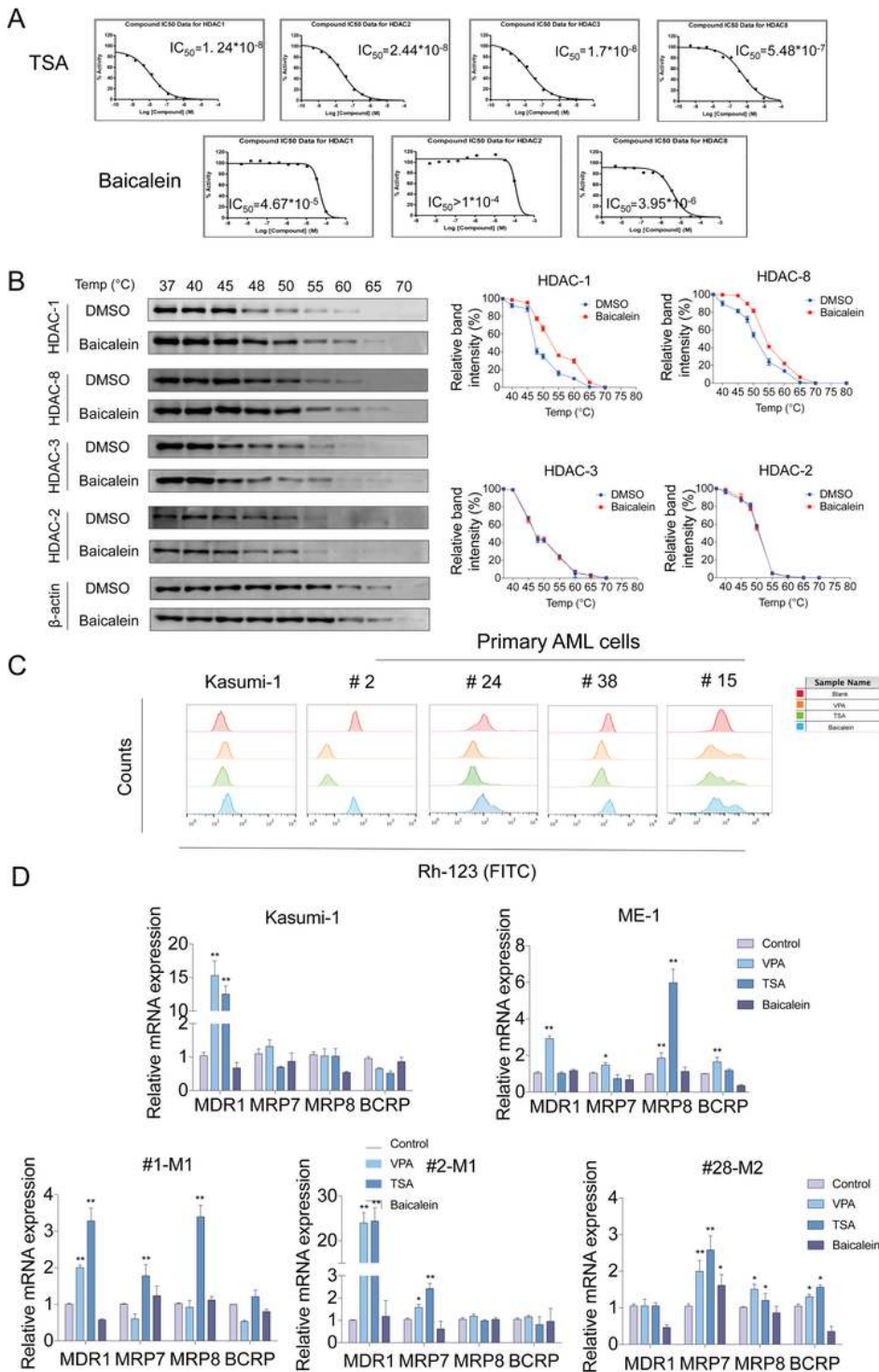
Figure 1

Effects of Baicalein on HDAC-1/8 activity and the expression of HDAC inhibitors-associated ABC transporter genes (A) HDACs Enzyme activity assay of Baicalein and TSA. (B) Quantification was made using western blot. ME-1cells were treated with Baicalein (30 μM) for 12 h, and temperatures between 37-70 °C and used to perform ITDRCETSA assay directed toward HDAC-1,HDAC-2,HDAC-3 and HDAC-8. Data were first normalized by setting the highest and lowest value in each set to 100 and 0%. Respectively.

Data were obtained in the presence of the Baicalein (red circle) as positive control and DMSO (blue square) as negative control. All experiments were performed at three independent occasions and data are given as the average ± SD. (C) Cells were respectively treated with 165 nM TSA or 3 mM VPA for 24 h or 30 μM Baicalein for 96 h. Medium as a negative control (Blank). The accumulation of Rhodamine 123 was detected by flow cytometry analyses. (D) Effects of various HDAC inhibitors on ABC transporter genes expression were analyzed by RT-qPCR. Cells were respectively treated with 165 nM TSA or 3 mM VPA for 24h or 30 μM Baicalein for 96 h. Medium as a negative control. Data represent the mean ± SD from three independent experiments. For analysis of RT-qPCR results, asterisks denote significant (*$P <$ 0.05 and **$P <$ 0.01) differences relative to controls by two-tailed Student's tests.
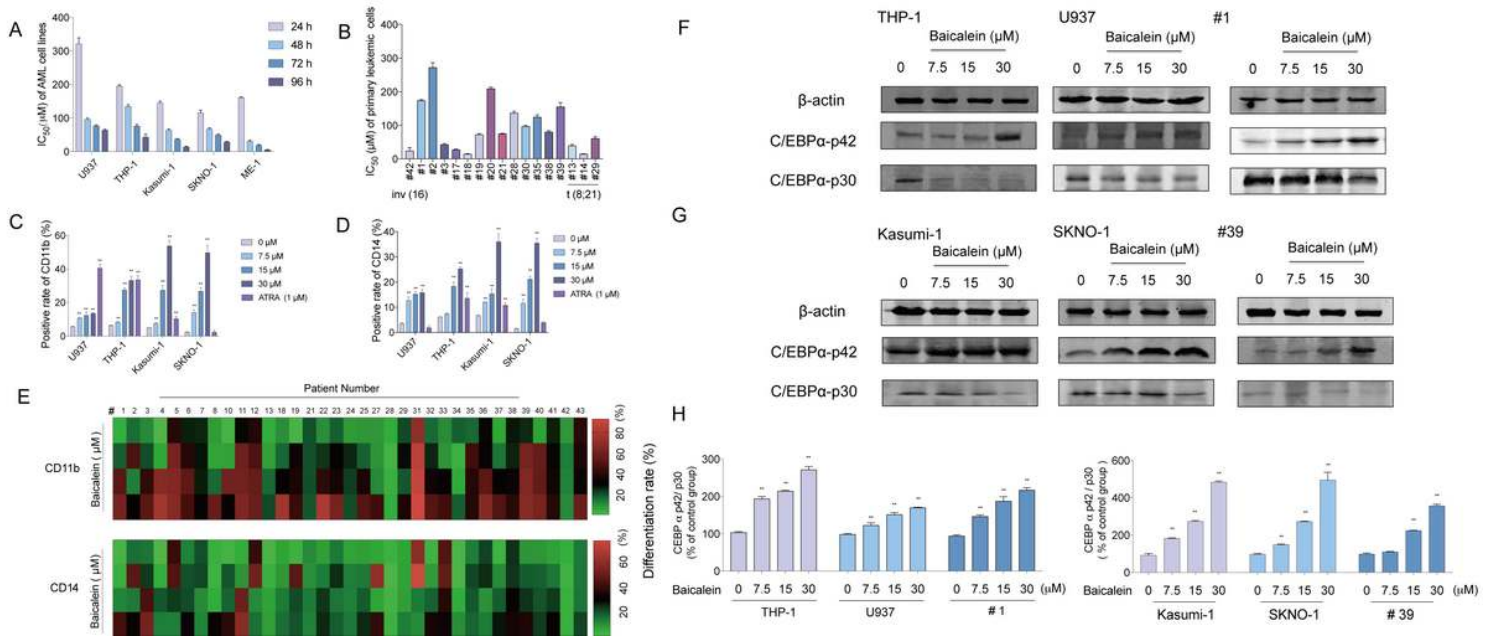

Figure 2

Effects of Baicalein on the growth and differentiation of AML cell lines and primary AML cells (A) The IC50 of Baicalein on AML cell lines after treatment for 24, 48, 72 and 96 h. (B) The IC50 of Baicalein on primary AML cells after treatment for 96 h. (C, D) The expression of CD11b/CD14 after Baicalein or ATRA treatment for 96 h in AML cell lines were shown. The percentages of cells expressing CD11b and CD14 were detected by flow cytometry analyses. The data represent the mean ± SD of 3 different experiments. Asterisks denote statistically significant (*$P <$ 0.05, ** $P <$ 0.01) differences compared with controls by one-way ANOVA. (E) Hot maps of the expression of CD11b/CD14 after Baicalein treatment for 96 h in primary AML cells are shown. The percentages of cells expressing CD11b and CD14 were detected by flow cytometry analyses. (F-H) Expression levels of C/EBPα-p42 and C/EBPα-p30 were analyzed by western blot after treatment with or without Baicalein for 96 h in AML cell lines and #1 and #39 AML samples. β-actin was used as a loading control. Those data represent the mean ± SD of 3 different experiments. Asterisks denote statistically significant *, $P <$ 0.05; **, $P <$ 0.01; differences compared with controls by one-way ANOVA.
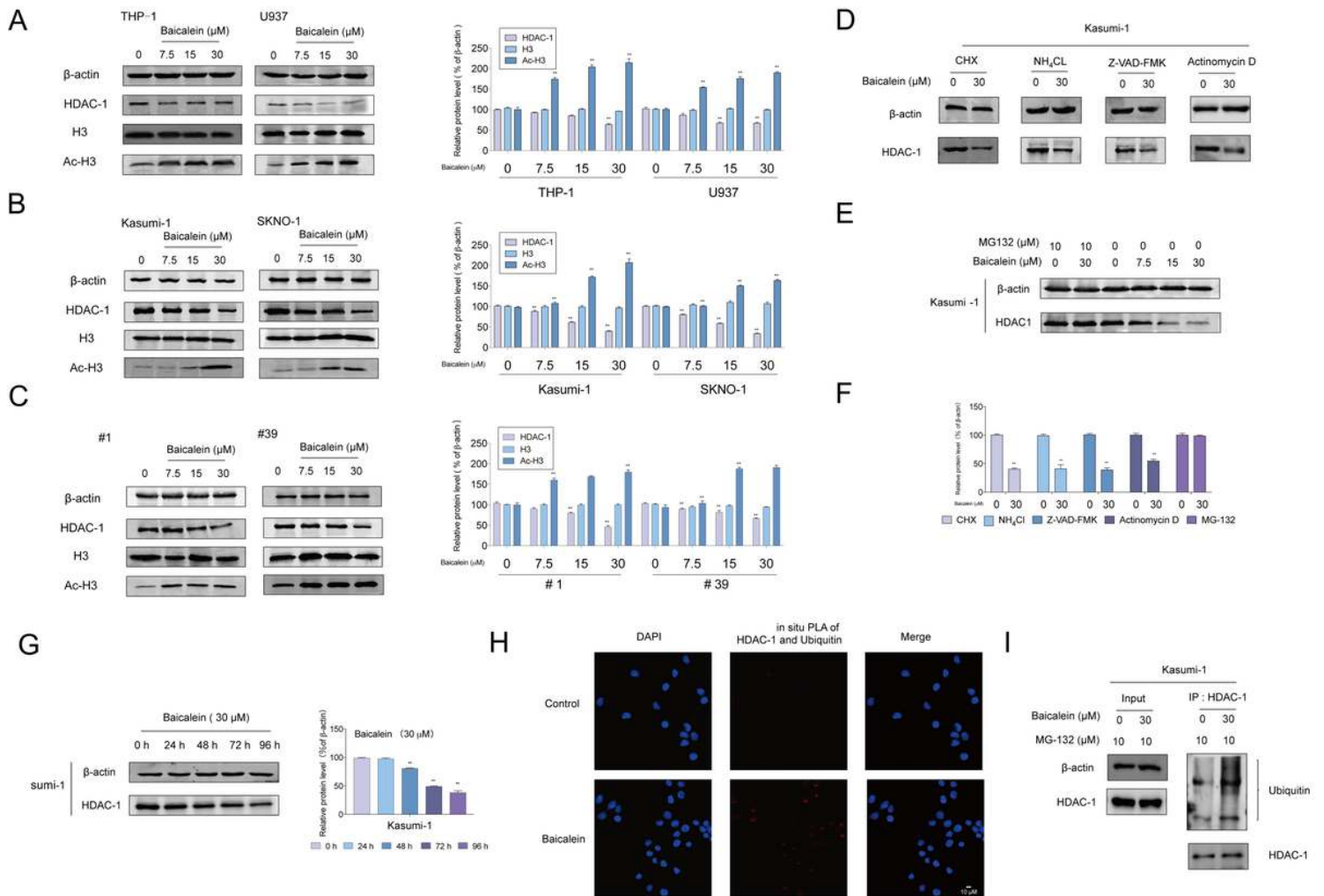
Figure 3

Baicalein promotes the degradation of HDAC-1 via proteasome-dependent pathway (A-C) Expression levels of HDAC-1, Histone-H3 (H3), and acetylated-Histone H3 (AC-H3) were analyzed by western blot after treatment with or without Baicalein for 96 h in AML cell lines and #1 and #39 AML samples. β-actin was used as a loading control. (D-F) Kasumi-1 cells were respectively treated with 15 μg/mL CHX or 5 mM NH4Cl or 20 μM z-VAD-FMK or 5 μg/mL Actinomycin D or 10 μM MG-132, and/or 30 μM Baicalein for 96 h. Western blot was performed to detect the expression of HDAC-1 with the indicated antibodies. (G) Kasumi-1 cells were treated with 30 μM Baicalein for 0, 24, 48, 72 and 96 h. Expression levels of HDAC-1 were analyzed by western blot. β-actin was used as a loading control. (H) Kasumi-1 cell were co-treated with 10 μM MG-132 and/or 30 μM Baicalein for 48 h. Then the co-IP of HDAC-1 and Ubiquitin was analyzed. Those results are representative of 3 independent experiments. (I) Representative images of Duolink in situ PLA using rabbit anti-Ubiquitin, mouse anti-HDAC-1 antibodies and PLA probes in Kasumi-1 cells after 30 μM Baicalein treatment for 48 h. Red foci indicate HDAC-1-Ubiquitin interactions, DAPI-stained nuclei are in blue. Those data represent the mean ± SEM of 3 different experiments. Asterisks

denote statistically significant *, P < 0.05; **, P < 0.01; differences compared with controls by one-way ANOVA.
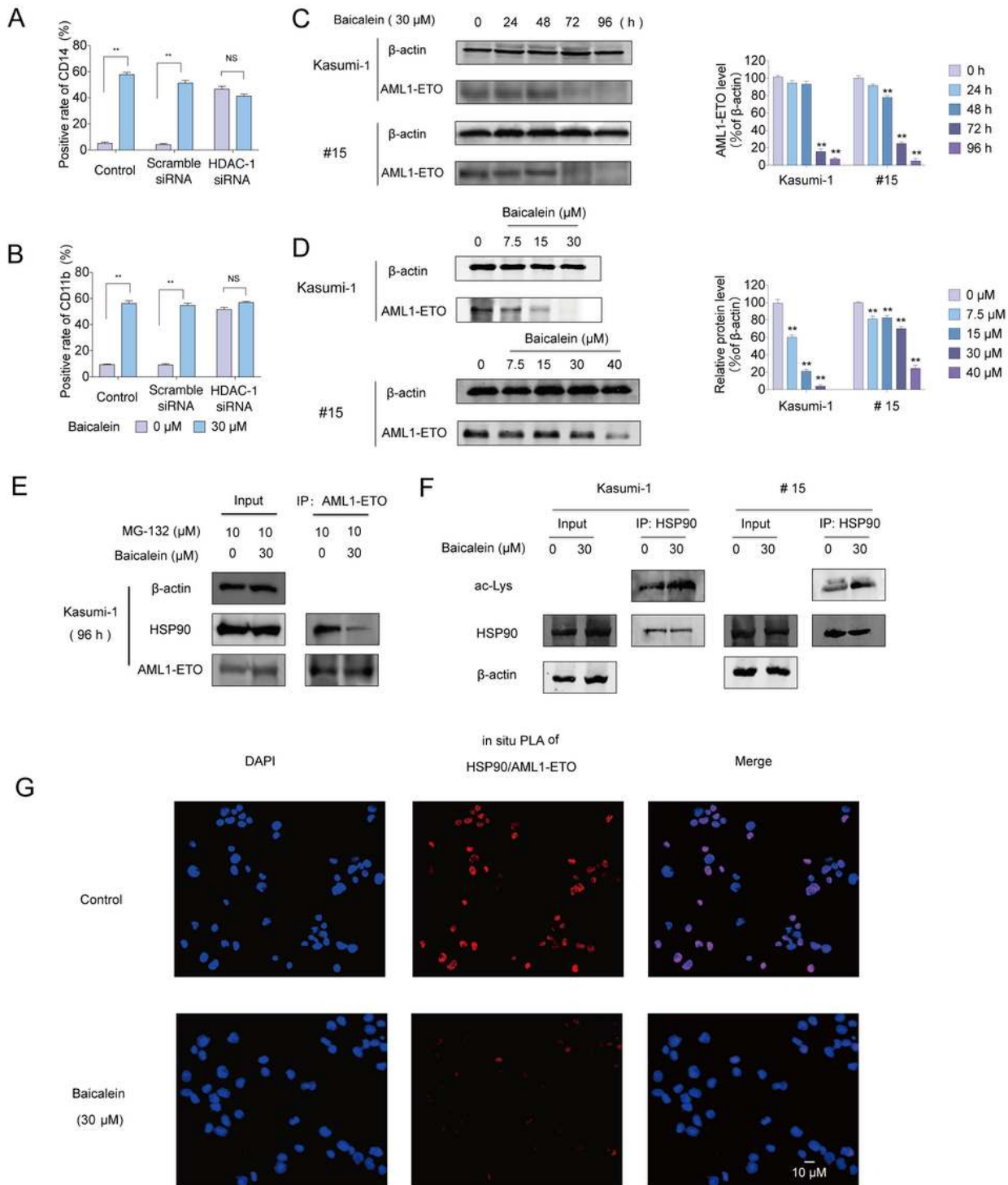

Figure 4

Baicalein interrupts the stability of AML1-ETO-HSP90 complex and induces differentiation of AML cells via HDAC-1 (A, B) The expression of HDAC-1 protein in Kasumi-1 cells was knocked down by HDAC-1 siRNA, and then the cells were treated with 30 μM Baicalein for 96 h. CD11b/CD14 expression were next

detected by flow cytometry analyses. CD11b and CD14 positive ratio of Kasumi-1 cells are shown. (C) Kasumi-1 and #15 sample were treated with 30 μM Baicalein for 0, 24, 48, 72 and 96 h. Expression levels of AML1-ETO were analyzed by western blotting. β-actin was used as a loading control. (D) Kasumi-1 and #15 sample were treated with or without different concentration of Baicalein for 96 h. Expression levels of AML1-ETO were analyzed by western blotting. β-actin was used as a loading control. (E) Kasumi-1 cells were co-treated with 10 μM MG-132 and/or 30 μM Baicalein for 48 h. Co-IP assay of HSP90 and AML1-ETO was analyzed. Western blot was performed with the indicated antibodies. β-actin was used as a loading control. (F) Co-IP assay of HSP90 and pan-Acetalyed-Lysine (Ac-Lys) in Kasumi-1 cells and #15 sample was performed after 30 μM Baicalein treatment for 48 h. (G) Representative images of Duolink in situ PLA using rabbit anti-HSP90, mouse anti-ETO antibodies and PLA probes after Baicalein treatment for 48 h in Kasumi-1 cells. Red foci indicate HSP90 and AML1-ETO interaction, DAPI-stained nuclei are in blue. Those data represent the mean ± SD of 3 different experiments. Asterisks denote statistically significant *, $P < 0.05$; **, $P < 0.01$; differences compared with controls by one-way ANOVA.
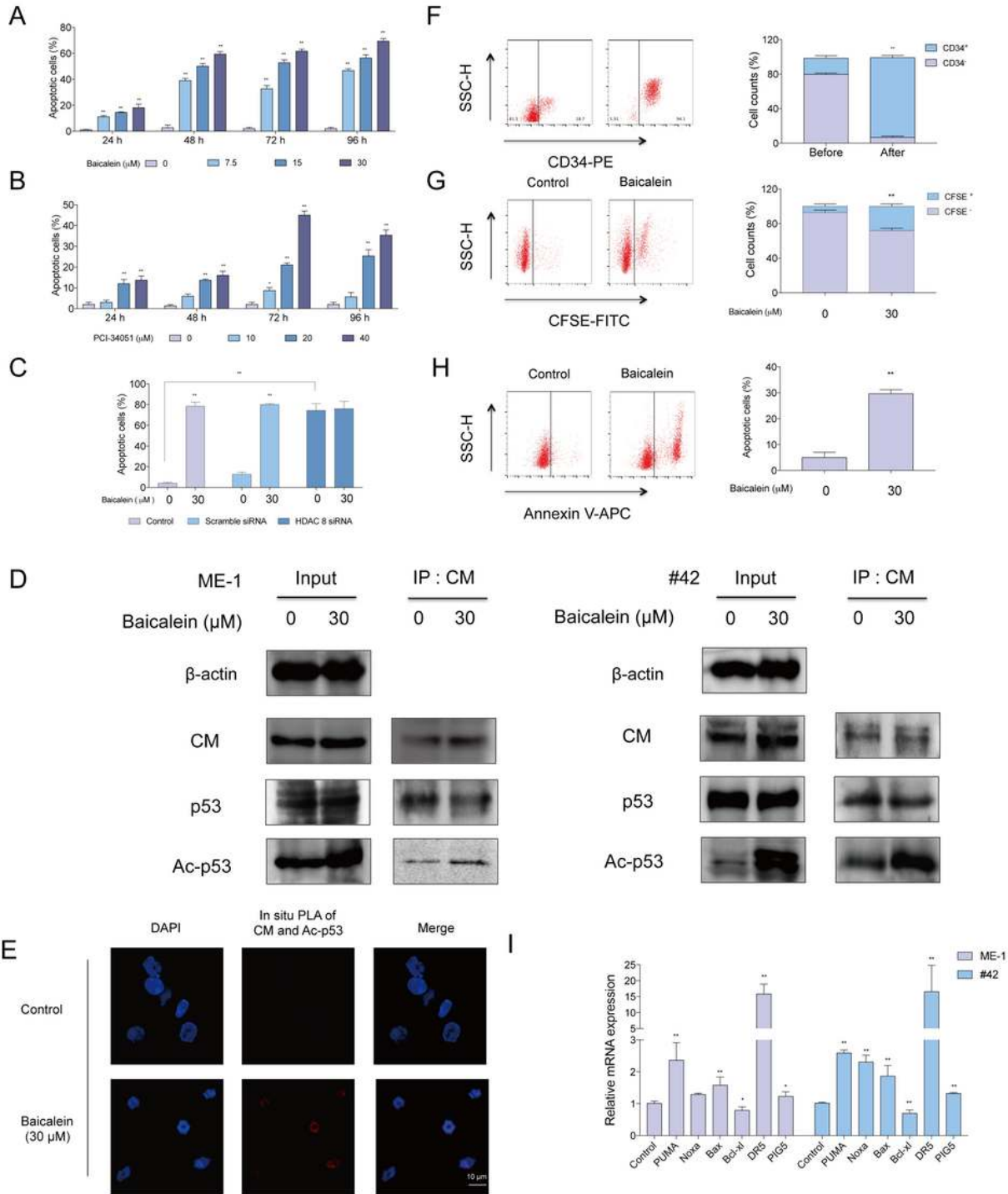
Figure 5

Effect of Baicalein on CM-p53-HDAC-8 complex and survival of CD34+ cells in inv(16) AML (A, B) Apoptosis effects of Baicalein on ME-1 cells. ME-1 cells were treated with DMSO, Baicalein (7.5, 15 and 30 μM) or PCI-34051 (10, 20 and 40 μM) for 24, 48, 72 and 96 h. Results were analyzed by flow cytometry. Data was representative of 3 independent experiments. (C) The expression of HDAC-8 protein in ME-1 cells was knocked down by HDAC-8 siRNA, and then the cells were treated with 30 μM Baicalein

for 48 h. Annexin V and PI expression of ME-1 cells were detected by flow cytometry analyses. Data was representative of 3 independent experiments. (D) Co-IP assay of p53 and CBF-β or Acetalyed-p53 (AC-p53) and CBF-β analysis in ME-1 cells and #42 sample were performed after Baicalein treatment for 24 h. (E) Representative images of Duolink in situ PLA using rabbit anti- CBF-β, mouse anti-AC-p53 antibodies and PLA probes were shown after Baicalein treatment for 24 h in ME-1 cells. Red foci indicate HDAC-1 and Ubiquitin interaction, DAPI-stained nuclei are in blue. (F) CD34+ cells were isolated from primary inv(16) AML cells (#42 sample) by MACS. CD34+ cells were gathered after magnetic separation. (G) CFSE staining assay of the purified CD34+ cells from #42 sample. The CD34+ cells were treated with DMSO or Baicalein (30 μM) for 48 h. The expression of CFSE was performed by flow cytometry. (H) Annexin V staining assay of the purified CD34+ cells from #42 sample. The CD34+ cells were treated with DMSO or Baicalein (30 μM) for 48 h. The expression of Annexin V was performed by flow cytometry. by flow cytometry. (I) Effects of Baicalein on the expression of downstream genes of p53 in ME-1 cells. Cells were treated with or without 30 μM Baicalein for 96 h, and then the genes expression was analyzed by RT-qPCR. Medium as a negative control. Those data represent the mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$;
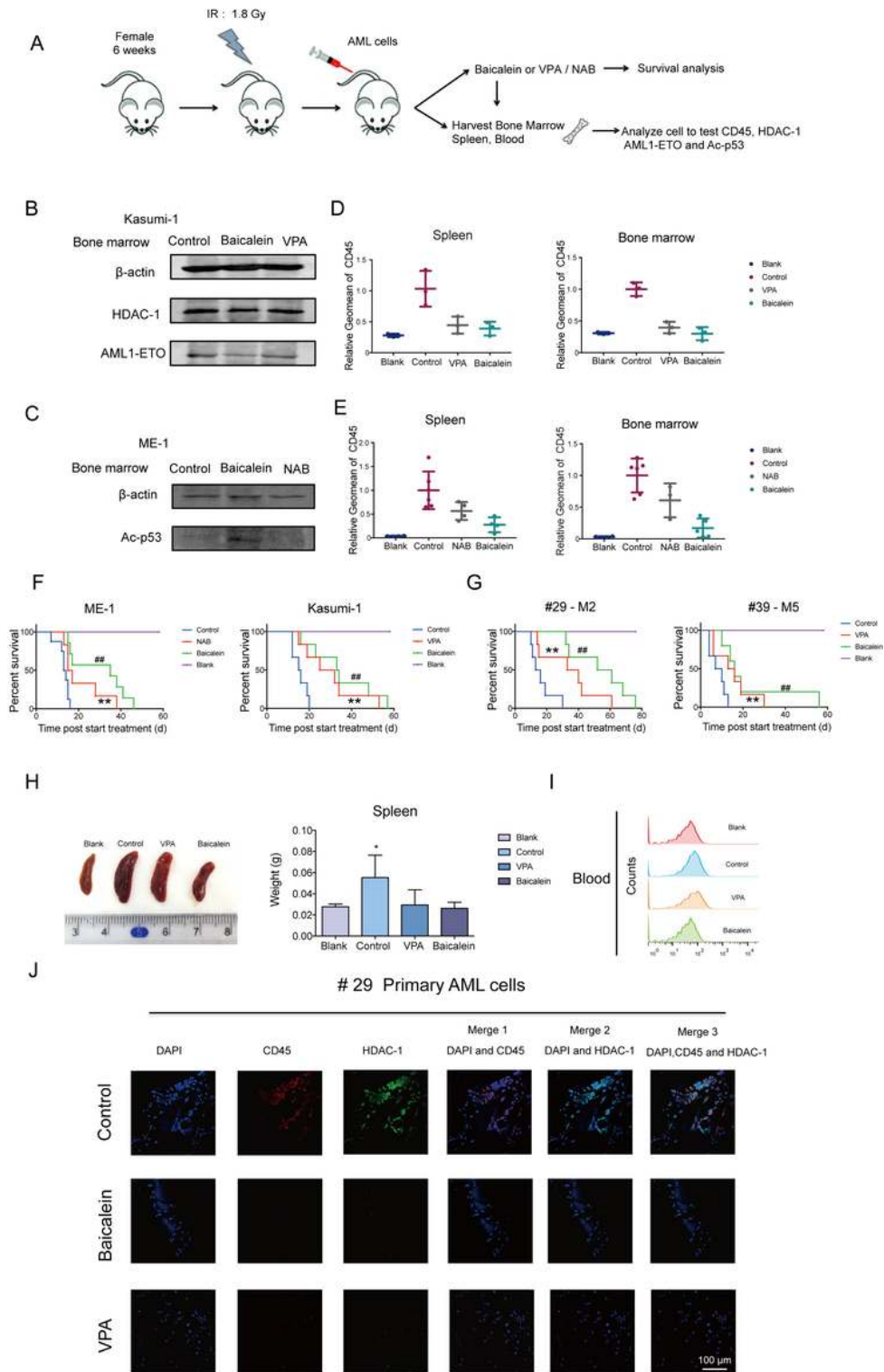
Figure 6

Anti-leukemic activity of Baicalein in AML cells-bearing NOD/SCID mice (A) Flowchart of evaluation the effect of Baicalein on AML cells-bearing NOD/SCID mice. (B, C) The expression levels of HDAC-1, AML1-ETO and Acetyl-p53(Ac-p53) in cells come from the Bone marrow of each group of AML cell lines-bearing mice were detected by western blot, β-actin was used as a loading control. (D, E) huCD45 expression were examined in cells come from Bone marrow and Spleen of each group of Kasumi-1 and ME-1 cells-bearing

mice by flow cytometry analyses. (F, G) A Kaplan-Meier survival plot for AML cells-bearing NOD/SCID mice was shown. (H) Effects of Baicalein and VPA on the weight of Spleen in different groups. And typical photos of spleen were shown. (I) huCD45 expression were examined in cells come from the blood samples from four mice (#29) of each group by flow cytometry analyses. (J) BM samples from three mice (#29) of each group were collected and sections were performed. Immunofluorescence and costained with huCD45-PE (red fluorescence) and anti-HDAC-1 (primary)/Alexa Fluor 488 donkey anti-goat (secondary) antibody combinations (green fluorescence), as well as DAPI (blue fluorescence). They were detected by confocal microscopy (FV1000; Olympus) with FV10-ASW2.1 acquisition software (Olympus) at room temperature (original magnification × 1000; immersion objective × 100/ × 60 with immersion oil type.

Supplementary Files

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- Supplementaryfiles.pdf