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Original Articles

Abivertinib, a novel BTK inhibitor: Anti-Leukemia effects and synergistic efficacy with homoharringtonine in acute myeloid leukemia

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ABSTRACT

Ibrutinib, an inhibitor of Bruton tyrosine kinase (BTK), has shown promising pharmacologic effects in acute myeloid leukemia (AML). In this study, we report that abivertinib or AC0010, a novel BTK inhibitor, inhibits cell proliferation, reduces colony-forming capacity, and induces apoptosis and cell cycle arrest in AML cells, especially those harboring *FLT3-ITD* mutations. Abivertinib was also found to be more sensitive than ibrutinib in treating AML. We demonstrate that in addition to targeting the phosphorylation of BTK, abivertinib also targeted the crucial PI3K survival pathway. Furthermore, abivertinib suppressed the expression of p-FLT3 and the downstream target p-STAT5 in AML cells harboring *FLT3-ITD* mutations. Moreover, *in vitro* and *in vivo* data revealed synergistic activity between abivertinib and homoharringtonine (HHT), a natural plant alkaloid commonly used in China, in treating AML cells with or without *FLT3-ITD* mutations. Collectively, these preclinical data suggest that abivertinib may be a promising novel agent for AML, with potential for combination treatment with HHT. Clinical studies on abivertinib-involved therapy are planned.

1. Introduction

Acute myeloid leukemia (AML) is the most common hematopoietic malignancy in adults, and is characterized by cytogenetic and genetic heterogeneity [1]. Clinical outcome remains poor for patients with AML, and there have been relatively fewer changes in the standard therapeutic approaches over the last 40 years [2]. Curative hematopoietic stem cell transplantation is the only recommended therapy for AML patients with poor prognosis, owing to severe morbidity and mortality associated with the disease [3]. Therefore, there is an urgent unmet need for novel AML therapies, including drugs targeting specific oncogenic proteins, epigenetic modulators, and immunotherapies. Following the approval of tyrosine kinase inhibitors, such as imatinib, which target BCR-ABL in chronic myeloid leukemia [4], researchers are committed to developing targeted treatments that improve the survival of AML patients.

Bruton tyrosine kinase (BTK) is a cytoplasmic protein which plays an important role in B-cell development. Initially, mutated BTK was discovered in the X-linked agammaglobulinemia, an immunodeficiency disease characterized by inhibition of B-cell maturation [5,6]. Ibrutinib is a first-in-class, orally and once daily administered, covalent inhibitor of BTK approved by the U.S. Food and Drug Administration and European Medicines Agency for the treatment of patients with mantle cell lymphoma (MCL) who have received at least one prior round of therapy. It has also been approved for the treatment of patients with chronic lymphocytic leukemia (CLL) who received at least one prior therapy, and for CLL patients who carried the 17p deletion. Moreover, ibrutinib has been approved by the Food and Drug Administration for the treatment of patients with Waldenström macroglobulinemia [7–13]. High expression of BTK is also reported in hematopoietic stem cells, and AML and ibrutinib can inhibit the proliferation of primary AML cells obtained from patients [14,15]. Abivertinib is a novel pyrrolopyrimidine-based irreversible inhibitor of epidermal growth factor receptor, which was also found to inhibit BTK (> 80%) at a concentration

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of 1 μM, with half maximal inhibitory concentration (IC_{50}) of 59 nM [16]. We have previously demonstrated the antitumor effects of abivertinib in MCL *in vitro*, which was related, in part, to the inhibition of BTK phosphorylation [17].

The aim of the present study was to investigate the potential utility of abivertinib as a novel treatment for patients with AML by investigating its antitumor effects in primary human AML blasts, cell lines, and xenograft models.

In this study, we demonstrate that abivertinib inhibited cell proliferation and reduced colony forming capacity in AML cells and that abivertinib was found to be more sensitive than ibrutinib. We also observed that abivertinib induced apoptotic cell death through downregulation of anti-apoptosis proteins, BCL-2, BCL-XL, and by activation of the caspase proteins. Meanwhile, cell cycle arrest at the G0/G1 phase was observed in AML cell lines and was proposed to be induced by downregulation of the phosphorylation of BTK and its downstream p-PLCγ-2. Moreover, we found that knockdown of BTK by siRNA could impair the sensitivity of abivertinib in AML cells. In addition to our finding that abivertinib is more sensitive than ibrutinib in treating AML, we found that abivertinib also targets the crucial PI3K survival pathway and suppresses the expression of p-FLT3 and downstream target p-STAT5 in AML cells with FLT3 mutations.

Homoharringtonine (HHT), a natural alkaloid derived from *Cephalotaxus*, is widely applied in AML therapy in China [18]. In our previous study, we demonstrated that Homoharringtonine-based induction regimen for patients with *de novo* AML resulted in better overall survival (OS) and superior 3-year event-free survival (EFS) to standard DA therapy in a multicenter, open-label, randomized, controlled phase 3 trial. However, adverse events were observed to be similar in all groups [19]. We also used HHT in combination with other drugs and obtained favorable results in *FLT3-ITD*-positive AML cell lines [20,21]. In this study, we demonstrate that abivertinib cooperates with HHT to inhibit the survival of AML cells via suppression of the PI3K pathway. Furthermore, the *in vivo* experiments confirmed that the combined therapy of abivertinib and HHT significantly prolonged survival in MOLM13 and MV4-11 AML xenograft model.

2. Materials and methods

2.1. Materials

Phosphorylated and total FLT3 (Tyr589/591), BTK (Tyr223), AKT (Ser473), STAT5 (Tyr694), PI3K (p110α), PLCγ2 (Tyr759), IKK (Ser176/180), NF-κB (Ser536), and CDK2, CDK4, CDK6, caspase-3, caspase-7, caspase-8, PARP, Bad, Bax, BCL-2, BCL-XL, and β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Human CD45 antibody was purchased from Abcam (Cambridge, MA, USA). Abivertinib was synthesized by Hangzhou ACEA Pharmaceutical Research Co., Ltd. Ibrutinib was purchased from Selleck Chemicals (Houston, TX, USA). HHT was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell lines and primary cells

MV4-11 and MOLM-13, human AML cells harboring FLT3 internal tandem duplication (ITD) mutations, were a kind gift from Professor Ravi Bhatia (City of Hope National Medical Center, Duarte, CA, USA) and were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS). KG-1, HL-60, U937, THP-1, and L-02 cell lines were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences. KASUMI-1 cell line was gifted by Professor Chen Saijuan (Shanghai Institute of Hematology, Shanghai, China). These cells were cultured in RPMI 1640 medium supplemented with 10% FBS.

Bone marrow and peripheral blood samples were obtained from AML patients following written informed consent. Mononuclear cells were isolated by Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation. Testing for *FLT3-ITD* was performed at the First Affiliated Hospital of Zhejiang University, Hangzhou, China. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University, China.

2.3. Colony forming assay

3% soft AGAR was diluted into 1% lower gum and 0.4% upper gum, respectively. AML cells were seeded in 6-well plates ($0.5–1 \times 10^3$ cells/well) in triplicate and treated with abivertinib (0.156 μM) for 14 days. Cell colonies were stained with 0.05% crystal violet solution for 30 min and counted.

2.4. Cell proliferation assay

Cells were seeded in 96-well plates ($1 \times 10^3–1 \times 10^4$ cells/well) in triplicate and treated with different drugs (abivertinib, ibrutinib, and HHT) for 24 or 48 h. Twenty microliters of MTS solution (Promega CellTitre96) (5 mg/mL) was added to each well followed by incubation for additional 4 h at 37 °C. Cell numbers were assessed based on the quantification of formazan by determining the absorbance at 490 nm.

2.5. Apoptosis assay

Induction of apoptosis was assessed using an apoptosis detection kit (BD Pharmingen, San Diego, CA, USA). After treatment with drugs for 24 or 48 h, the cells were washed twice with phosphate buffered saline (PBS), resuspended in binding buffer, and incubated with Annexin V-FITC and Propidium Iodide (PI) for 15 min. Apoptotic cells were analyzed by flow cytometry using FACScan™ flow cytometer (Becton Dickinson, San Diego, CA, USA).

2.6. Cell cycle analysis

After treatment with drugs for 24 or 48 h, cells were harvested and fixed overnight with 75% ethanol at 4 °C, followed by two PBS washes and incubation in buffer containing 50 μg/mL PI and 100 μg/mL RNase A for 30 min at room temperature. Cell cycle analysis was conducted using FACScan™ flow cytometer (Becton Dickinson).

2.7. Western blot analysis

Cells were lysed in radioimmunoprecipitation (RIPA) buffer (Cell Signaling Technology) on ice for 30 min. Protein concentration of the cellular supernatant was determined using BCA reagent after centrifugation of the cell lysate at 12000 $\times g$ for 15 min at 4 °C. Western blotting was performed after 10% SDS-PAGE (Life Technologies, Carlsbad, CA, USA), with the cellular proteins transferred onto a pre-activated PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h and incubated with primary antibodies overnight at 4 °C. After incubation with primary antibody, the blots were washed thrice with TBST buffer, and membranes were incubated with secondary antibodies (Cell Signaling Technology) for 1 h at room temperature. The target proteins were visualized using an ECL detection kit (Amersham, Little Chalfont, UK) and analyzed using Image Lab™ software (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. RNA interference

The siRNAs against BTK were purchased from Invitrogen and were transfected into KASUMI-1 and MV4-11 cells using Invitrogen™ Lipofectamine™ RNAiMAX (Fisher Scientific, Pittsburg, PA, USA), according to the manufacturer's instructions. In brief, the cells were seeded at the density of 5×10^5/well in 6-well plates. siRNA (200 nM)

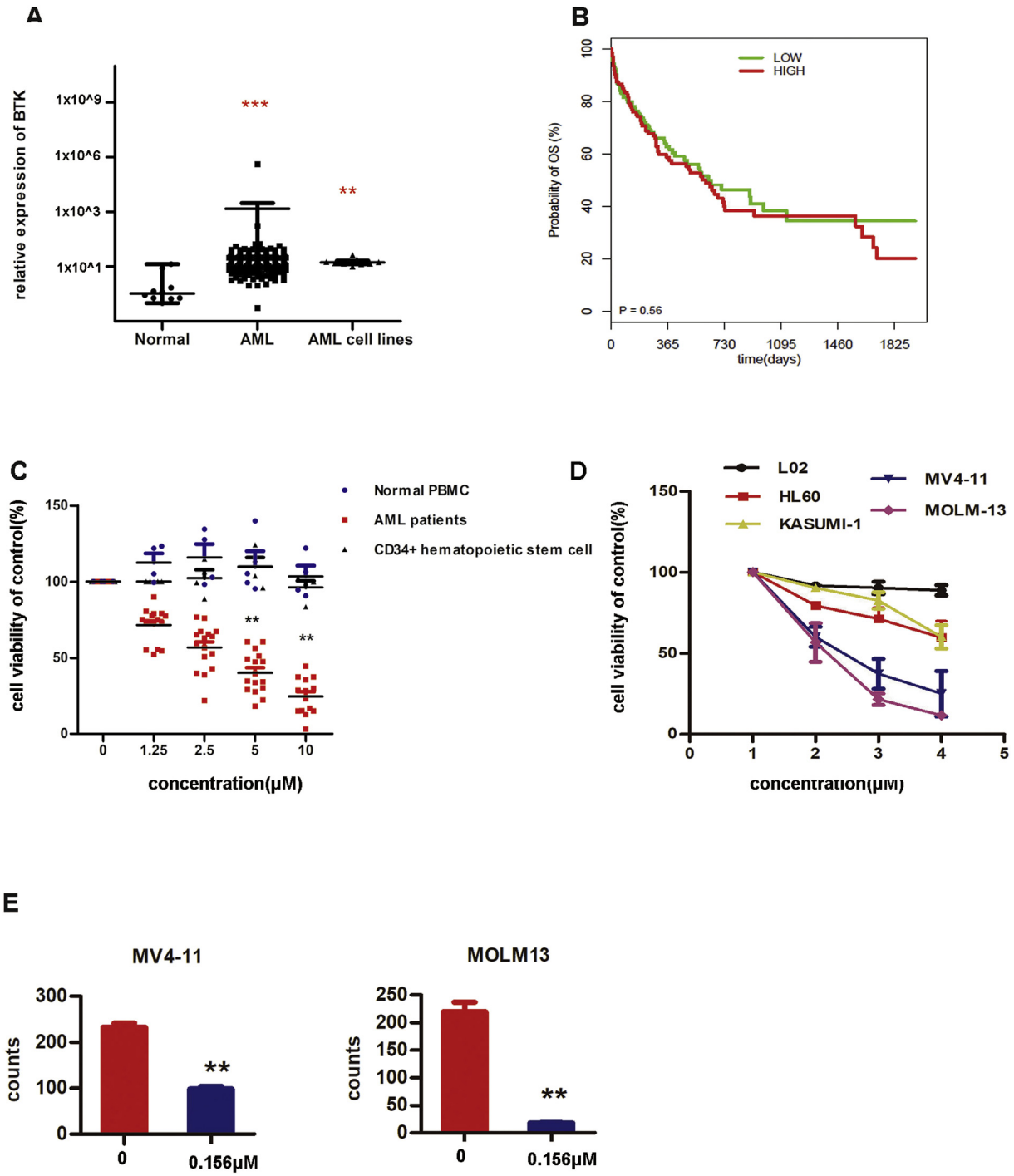

Fig. 1. Abivertinib inhibits cell survival in AML (A) *BTK* mRNA expression in primary AML cells from 279 patients, 9 AML cell lines and PBMCs from 9 healthy donors, $p < 0.0001$. (B) Kaplan-Meier analysis of overall survival according to *BTK* mRNA expression in primary blasts from 279 AML patients, $p = 0.56$. (C) Fifthteen AML blasts (including one harboring *BTK* FLT3-ITD mutation), PMMCs from healthy controls (n = 4) and CD34 positive hematopoietic stem cell from cord (n = 4) were treated with increasing doses of abivertinib (1.25–10 μM) for 24 h and then cell proliferation assessed (MTS assay), $p = 0.0095$. (D) AML cell lines were treated with increasing doses of abivertinib (1–4 μM) for 24 h and cell proliferation assessed (MTS assay). Data were normalized to DMSO-treated cells and represent the mean (SD) of 3 experiments. (E) Colony-forming assays in AML cell lines, MV4-11 and MOLM13 were performed to show the number of colonies or colony-forming cells (CFC). Data were normalized to DMSO-treated cells ($p = 0.0052$ and 0.0075).

and 10 μL Lipofectamine were mixed in 500 μL Opti-MEM (Fisher Scientific) and incubated at room temperature for 20 min before adding the transfection mix to cells. Cells were then incubated at 37 °C for 72 h.

2.9. RNA extraction and real time PCR (qRT-PCR)

Total RNA was extracted from the cells using TRIzol reagent according to manufacturer's instructions. Reverse transcription was performed using RNA PCR core kit (Life Technologies, Paisley, UK). Quantitative real-time PCR was carried out using SYBR Green qPCR mastermix and GAPDH was used as internal control. The sequences of the primers were as follows: GAPDH forward, 5′- GGAGCGAGATCCCT CCAAAAT -3′ and reverse, 5′- GGCTGTTGTCATACTTCTCATGG -3'; BTK forward, 5′- TCTGAAGCGATCCCAACAGAA- 3′ and reverse, 5′- TGCA

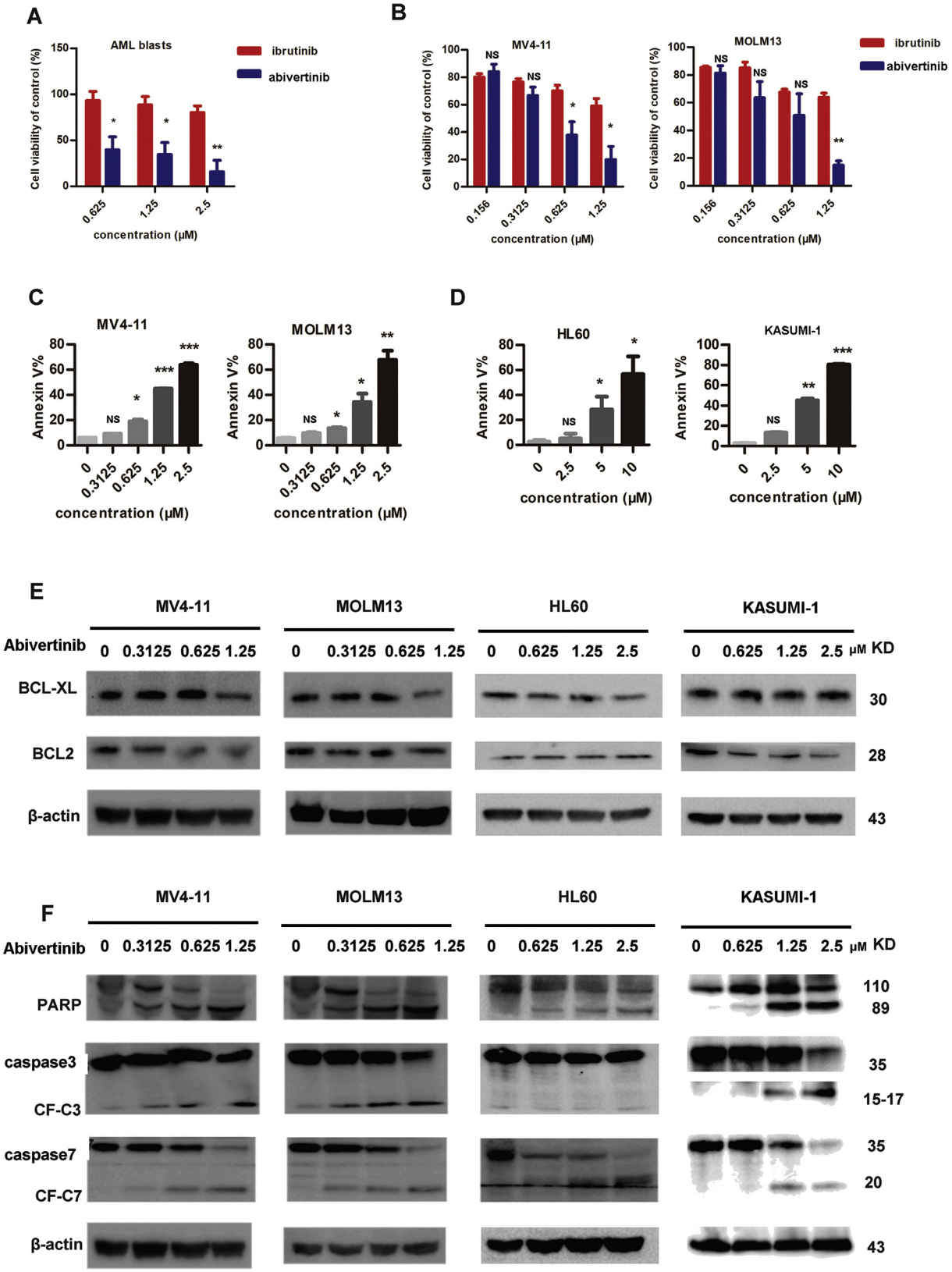

Fig. 2. Abivertinib induces apoptosis of AML cells. (A) Four AML blasts were treated with same doses of abivertinib or ibrutinib respectively for 24 h and cell viability assessed (p = 0.0354, 0.0144, 0.0041). (B) AML cell lines (MV4-11 and MOLM13) were treated with increasing doses of abivertinib or ibrutinib for 24 h followed by MTS assay p = 0.0019. (C) MV4-11/MOLM13 (D) KASUMI-1/HL60 were treated with increasing doses of abivertinib for 24 h, cells were co-stained with Annexin V and PI and apoptosis was measured by FCM. Expression of (E) BCL-2, BCL-XL, and (F) PARP, caspase-3 and -7 were analyzed by Western blotting analyses in cell lines referred above after treated with increasing dose of abivertinib for 6 h and 24 h respectively. * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$.

CGGTCAAGAGAAACAGG -3'. The Y-axis in Fig. 1A represents the relative expression of BTK, and the expression level of THP-1 cells was used as reference standard.

2.10. AML xenograft model

Severe combined immunodeficiency (SCID) and NOD-SCID gamma (NSG) mice were purchased from Shanghai SLRC Laboratory Animal Center. Our animal study was approved by Ethics Committee for Laboratory Animals of the First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were exposed to a 10/14 h light-dark cycle, kept under normal room temperature and fed by standard pellet food and tap water.

For the MOLM13-SCID mice xenograft model, 10×10^6 MOLM13 cells were injected into 5-week-old SCID mice via the tail vein. After 7 days, the mice were randomly assigned to 1 of 5 groups: 0.5% methyl cellulose (MC, day 8–37, PO), 50 mg/kg abivertinib (day 8–37, PO), 100 mg/kg abivertinib (day 8–37, PO), 0.25 mg/kg HHT (day 8–14, intraperitoneal), or 50 mg/kg abivertinib and 0.25 mg/kg HHT (administered per the single-agent groups). The mice were humanely sacrificed after observation of typical leukemic symptoms. PBMCs, bone marrow, and spleen samples were harvested for flow cytometry analysis. Human CD45 antibody was used to identify engraftments.

For the MV4-11-luc-NSG xenograft model (Bitocytogen, China), 2×10^6 MV4-11 luc cells (a gift from Dr. Xu [The Second Affiliated Hospital of Zhejiang University, Hangzhou, China]) were injected into the NSG mice via the tail vein. After 7 days, cell engraftment was assessed following injection of D-luciferin (150 mg/kg, intraperitoneally) using IVIS-2® Imaging System (Xenogen, Alameda, CA, USA). Mice were randomly assigned to 5 groups according to the intensity of the D-luciferin signal. The 5 groups were treated as described for the MOLM13 SCID xenograft model above. Engraftment analysis was carried out every week using the D-luciferin method.

2.11. Statistical analyses

Data were analyzed using GraphPad Prism 6.0 software. Summary statistics (mean ± SD) are represented with statistical significance assessed using Mann-Whitney test ($p < 0.05$ was considered statistically significant). Survival was analyzed using the Kaplan-Meier method and analyzed using a log rank test.

3. Results

3.1. Abivertinib inhibits cell proliferation and reduces colony forming capacity of AML cells, especially with FLT3-ITD mutation

BTK mRNA expression was significantly higher in primary AML samples (n = 279) and 9 AML cell lines than in PBMCs obtained from healthy donors (n = 9; $p < 0.0001$ and $p < 0.0001$; Fig. 1A). However, *BTK* mRNA expression did not appear to be a prognostic marker, since the OS was similar in AML patients (n = 279) grouped according to high (n = 136) and low (n = 143) *BTK* mRNA expression levels ($p = 0.56$; Fig. 1B).

We next investigated the impact of increasing the dose of abivertinib (1.25–10 μM) for 24 h on the viability of primary AML cells from 15 patients (n = 1, with the *FLT3-ITD* mutation) and PBMCs from four healthy donors. MTS assay for cell proliferation revealed that abivertinib significantly inhibited the viability of primary AML blasts compared to the PBMCs from healthy donors ($p = 0.0095$; Fig. 1C). While abivertinib negatively impacted the viability of AML blasts in a concentration-dependent manner, none of the tested concentrations affected the viability of PBMCs from healthy donors and $CD34^+$ hematopoietic stem cells from the umbilical cord blood (Fig. 1C). Reduction in viability of the AML cell lines (KASUMI-1, MOLM13, MV4-11, and HL60) treated with abivertinib (1–4 μM) for 24 h were similar to those observed for AML blasts (Fig. 1D). Furthermore, while abivertinib suppressed the proliferation of AML cell lines, with MV4–11 and MOLM13 cells demonstrating greatest sensitivity, abivertinib did not appear to affect the proliferation of a normal liver tissue cell line, L-02 (Fig. 1D). Abivertinib treatment (0.156 μM) for 14 days also reduced colony formation capacity in MV4-11 and MOLM13 cells (Fig. 1E).

3.2. AML cells are more sensitive to abivertinib compared with ibrutinib

To compare the effects of abivertinib and ibrutinib on AML cells, four primary AML blast cell lines were treated with abivertinib or ibrutinib (0.625–2.5 μM) for 24 h. Cell proliferation examined by MTS assay revealed significantly lower cell growth with abivertinib versus ibrutinib at each dosage level ($p \leq 0.035$, 0.0144, and 0.0041; Fig. 2A). MV4-11 and MOLM13 cell lines also demonstrated greater sensitivity to abivertinib versus ibrutinib in terms of reduced cell viability at doses of 0.625 μM and 1.25 μM, respectively ($p \leq 0.0019$; Fig. 2B).

3.3. Abivertinib induces apoptosis in AML cell lines

The effect of abivertinib on induction of apoptosis in AML cell lines was investigated by flow cytometry analysis. In MV4-11 and MOLM13 cells, dose-dependent induction of apoptosis was observed upon abivertinib treatment at concentrations of 0.625–2.5 μM for 24 h (Fig. 2C). Abivertinib-induced apoptosis was also observed in KASUMI-1 and HL60 cells, albeit at higher drug concentrations (5–10 μM; Fig. 2D).

To investigate how abivertinib affects apoptosis in AML cells, we measured the levels of apoptosis-related proteins. When MV4-11, MOLM13, KASUMI-1, and HL60 cells were treated with abivertinib for 6 h, downregulation of antiapoptotic proteins, BCL-2, BCL-XL (Fig. 2E) and MCL-1(supplymentary Fig. 3A) was observed. When the treatment time was extended to 24 h, the levels of cleaved caspases, caspase-3, caspase-7, and cleaved-PARP increased in a dose-dependent manner (Fig. 2F).

3.4. Abivertinib induces cell cycle arrest at G0/G1 stage in AML

The effects of abivertinib on the cell cycle progression were investigated using flow cytometry. Treatment of MV4-11 and MOLM13 cells with abivertinib for 24 h significantly inhibited cell cycle progression at G0/G1 in a dose-dependent manner up to 0.625 μM (Fig. 3A). At concentrations over 0.625 μM, the cycle arrest effect of abivertinib was reversed but also existed (Fig. 3A). This finding suggests that the inhibitory effects of abivertinib on AML cells may be associated with cell cycle arrest at lower doses, while apoptosis predominates at higher concentrations. In KASUMI-1 and HL60 cell lines, no apparent cell cycle alterations were observed at the same dosage treatment while G0/G1 phase arrest was noted when the concentration was increased to 5 μM, as shown in Fig. 3A and supplementary material. Next, we examined the levels of cell cycle-dependent kinases after treatment with different concentrations of abivertinib in AML cell lines, and observed that levels of CDK2, CDK4, and CDK6 declined significantly in a concentration-dependent manner (Fig. 3B), consistent with the flow cytometry results. These results indicate that abivertinib inhibited cell cycle progression from G0/G1 to S phase via downregulation of CDKs in AML cell lines.

3.5. Abivertinib inhibits BCR signaling in a BTK-dependent or -independent manner in AML

To confirm if abivertinib targets BTK, we investigated the impact of

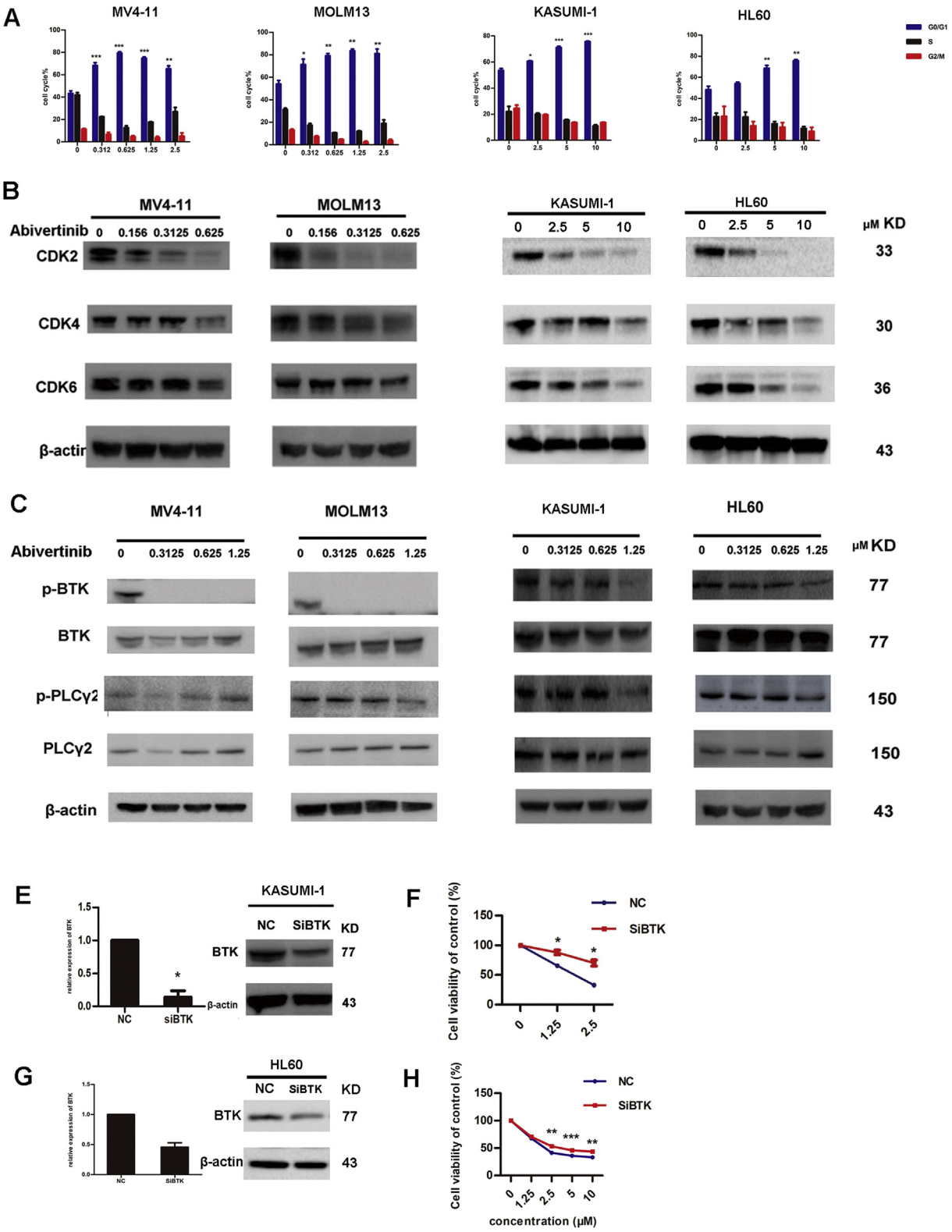

Fig. 3. Abivertinib arrested cell cycle at G0/G1 phase in AML cells. (A) AML cell lines were treated with increasing doses abivertinib for 24 h. The cells were stained with propidium iodide and underwent FCM analysis to determine cell cycle distribution. (B) Soluble proteins CDK4, CDK6, CDK2 of AML cell lines were analyzed by Western blotting analyses at the indicated concentrations for 24 h. (C) abivertinib inhibited AML proliferation via BTK-dependent manner. AML cell lines were treated with increasing dose abivertinib for 24 h. Western blot analysis was conducted for P-BTK-Y556, total BTK, p-PLCγ2, and total PLCγ2. (D) mRNA and protein expression of BTK after knocked down by siBTK in KASUMI-1 compared with negative control(NC) KASUMI-1 cells($p = 0.0123$). (E) The inhibitory effect of abivertinib on cell viability (MTS assay) in KASUMI-1 cells with knocked down of BTK expression compared with NC KASUMI-1 cells ($p = 0.0205$). * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$.

increasing abivertinib concentrations (0.3125–1.25 μM) on the B-cell antigen receptor (BCR) signaling pathway, including phosphorylation of total BTK (p-BTK) and downstream PLCγ2 (p-PLCγ2) in AML cell lines, with or without *FLT3-ITD* mutation. As expected, the levels of p-BTK and p-PLCγ2 proteins were downregulated, while total BTK and PLCγ2 levels remained unchanged (Fig. 3C). These results indicated that abivertinib treatment could inhibit the abnormalities in the BCR signaling pathway in AML cells.

To confirm that abivertinib inhibited the proliferation of AML cells by targeting BTK, siRNAs were used to knock down BTK in AML cells. Reduced BTK expression in the knock-down cells was confirmed by qPCR and western blotting (Fig. 3E and G). When BTK-knockdown KASUMI-1 and HL60 cells were treated with abivertinib (1.25 μM–2.5 μM and 1.25 μM–10 μM) for 48 h, the inhibitory effect of abivertinib on the viability of AML cells was lower than that in negative control cells ($p < 0.05$; Fig. 3F and H). In contrast, a reduction in the inhibitory effects of abivertinib on cell viability upon knockdown of BTK was not observed in MV4-11 and MOLM13 cells (Supplementary Fig. 1A-D). These observations suggest that in addition to an effect on BTK signaling, there may be other mechanisms by which abivertinib inhibits the survival of AML cells.

To investigate the potential mechanisms involved, we analyzed the expression levels of PI3K and its downstream signaling proteins, p-AKT, p-IKK, and p-NF-κB in the four AML cell lines upon abivertinib treatment. Interestingly, we found that the PI3K signaling pathway axis was significantly inhibited after drug exposure as shown in Fig. 4A.

3.6. Abivertinib affects FLT3-ITD-mediated signaling in drug-sensitive cell lines

The effect of abivertinib on FLT3-ITD-mediated signaling in abivertinib -sensitive AML cell lines, MV4-11 and MOLM13, was investigated. Abivertinib potently inhibited p-FLT3 and p-STAT5 protein expression (Fig. 4B). MV4-11 cells also revealed lower expression of p-STAT5 upon treatment with abivertinib as compared with an equivalent concentration of ibrutinib (Fig. 4C).

3.7. Abivertinib inhibits the proliferation of primary AML blasts by BTK-dependent and -independent mechanisms

To further investigate how abivertinib affects the signaling pathways relevant in the development of AML, primary AML blasts from two patients were cultured with different concentrations of abivertinib (Fig. 4D and E). Protein levels of p-BTK and p-PLCγ2 were downregulated and expression of PI3K and downstream p-AKT and p-IKK was also downregulated, similar to that observed in the AML cell lines. Together, these results confirmed that abivertinib inhibited the proliferation of AML cells by BTK-dependent and independent pathways *in vitro*.

3.8. Combined treatment of abivertinib with HHT inhibits AML cell survival

A combined treatment of abivertinib and HHT was associated with promising antiproliferative effects (MTS assay) in AML blasts obtained from all six donors (AML#1 harbored *FLT3-ITD* mutation). The effect of the combined treatment exceeded that of single-agent HHT and was similar to or greater than that observed with single-agent abivertinib (Fig. 5A). AML cell lines also demonstrated promising anti-tumor effects when cultured with HHT plus abivertinib, including those harboring (MV4-11, MOLM13) or without (KASUMI-1) *FLT3-ITD* mutation (Fig. 5B).

Abivertinib plus HHT treatment also induced significantly more apoptosis in MV4-11 and MOLM13 cell lines compared with abivertinib or HHT alone for 24 h or 48 h (all $p < 0.0001$ in Fig. 5C). Expression of the anti-apoptotic proteins, BCL-2 and BCL-XL, was also found to be lower when cells were treated with abivertinib plus HHT (Supplementary Fig. 1C). Moreover, expression of cleaved caspase-3 and cleaved caspase-8 was higher in cells cultured with single agents compared with that observed for abivertinib plus HHT (Supplementary Fig. 1C).

Combined treatment of HHT and abivertinib demonstrated a clear synergistic effect on downregulation of PI3K and its downstream effector p-AKT in MV4-11 and MOLM13 cells (Supplementary Fig. 1D).

3.9. Synergistic anti-leukemia effect of abivertinib plus HHT in vivo

The effects of abivertinib *in vivo* were investigated in MV4-11-luc-NSG xenograft mice. All the drug administrations referred to started 7 days after injection of MV4-11-luc cells, when leukemia cells engrafted in the bone marrow. All mice had equal tumor burdens, measured by photon intensity at the beginning of the therapy (Supplementary Fig. 5A). Mice treated with combination of abivertinib (50 mg/kg) and HHT (0.5 mg/kg) had significantly lower leukemia tumor burden after 14 days compared with those treated with vehicle or abivertinib alone ($p < 0.05$; Fig. 6A). We also detected the human-CD45$^+$ cells in mouse bone marrow, as shown in Fig. 6B. The engraftment in the combination group was significantly lesser than that observed with the vehicle or agent alone. Weight of the mice was assessed every three days to assess treatment tolerance. At Day 21, mice receiving combination therapy or single-agent treatment experienced lesser weight loss than those receiving vehicle alone (Fig. 6C).

Abivertinib (50 mg/kg) did not prolong time till hemiplegic paralysis which indicated leukemia cells severely invade the bone marrow in MV4-11-luc-NSG mice. However, while the higher dose of abivertinib (100 mg/kg) and HHT (0.5 mg/kg) failed to decrease leukemia burden, it resulted in a significantly increased survival advantage when compared to that observed with the vehicle ($p < 0.05$). Furthermore, mice receiving abivertinib plus HHT experienced significantly prolonged survival than mice receiving both agents individually ($p < 0.05$ and $p < 0.01$; Fig. 6D).

Similar results were observed in MOLM13-SCID xenograft mice. Expression of human-CD45$^+$, the surface marker of bone marrow cells, was analyzed to confirm that the AML xenografts were established (Supplementary Fig. 5B). Only Abivertinib (50 mg/kg) did not significantly prolong the survival of MOLM13-mice when compared with the vehicle treatment. However, abivertinib at a higher dose of 100 mg/kg or combined with HHT resulted in a longer survival time when compared with the vehicle group ($p < 0.05$; Supplementary Fig. 5C). Hence, the *in vivo* study confirmed the *in vitro* observations that abivertinib enhances the anti-leukemia effect of HHT.

4. Discussion

BTKs play a crucial role in normal B-cell differentiation and hematopoietic signaling and are widely expressed in hematologic cells with the exception of T-cells [22–24]. Rushworth et al. reported that BTK was constitutively phosphorylated in AML cells [14]. Our study demonstrates that expression of *BTK* mRNA in primary AML cells from 279 patients was significantly higher than that observed in PBMCs from 9 healthy donors, consistent with previously reported data [25].

While high expression of *BTK* was not significantly correlated with unfavorable prognosis in AML patients in our study, targeting BTK with ibrutinib has been previously shown to effectively inhibit AML clone formation [14]. In our study, we observed that abivertinib also effectively inhibited the survival and colony formation in AML cells. All primary AML cells, including those obtained from a patient with *FLT3-ITD* mutation, were sensitive to abivertinib. Among the AML cell lines studied, MV4-11 and MOLM13 cells that carried *FLT3-ITD* mutation showed higher sensitivity to abivertinib. These findings indicate that abivertinib can effectively inhibit AML cell proliferation and that AML cells with *FLT3-ITD* mutation, which is associated with poor prognosis, are sensitive to abivertinib. It is also noteworthy that we observed a

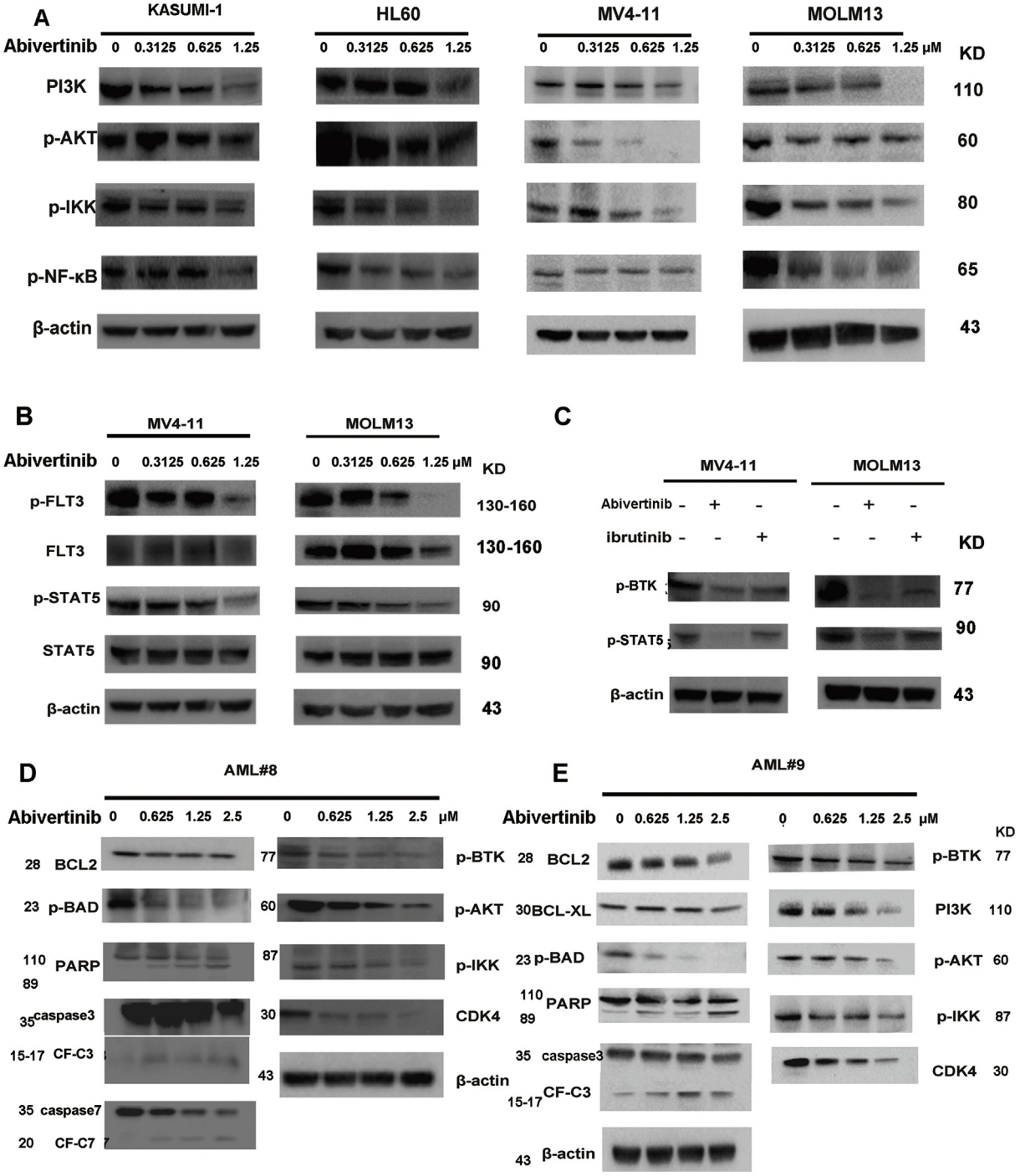

Fig. 4. (A) Abivertinib inhibits the survival of AML cell-lines by a BTK-independent manner. AML cell lines were treated with increasing doses of abivertinib for 24 h. Western blot analysis was conducted to examine PI3K, p-AKT-S473, P-IKK, p-NF-κB protein levels. Abivertinib downregulated the FLT3 signal pathway. (B) MV4-11 and MOLM13 cells were treated with increasing doses of abivertinib for 24 h. Western blot analysis was conducted for P-FLT3, total FLT3, p-STAT5, total STAT5 protein levels. (C) MV4-11/MOLM13 were treated with 0.3125 μM of abivertinib or ibrutinib respectively for 6 h followed by Western blot analysis of p-BTK and p-STAT5. (D) Primary AML#8 and (E) AML#9 cell from were treated with same doses of abivertinib for 24 h followed by Western blot analysis of proteins referred above.

lower IC_{50} for abivertinib compared with that for ibrutinib in MV4-11 and MOLM13 cells, suggesting that abivertinib is more active than ibrutinib in AML cells. This observation may be explained, in part, by higher downregulation of BTK protein in cell lines treated with abivertinib as compared with ibrutinib.

In this study, we also noted that abivertinib downregulated BCR signaling, p-BTK and p-PLCγ2 levels, while total BTK and PLCγ2 levels remained unchanged. These findings were further investigated by knocking down BTK expression in KASUMI-1 and MV4-11 cells using siRNAs against BTK. Suppression of survival induced by abivertinib was impaired in KASUMI-1 cells but not in MV4-11 cells. These results indicated that abivertinib may exert anti-leukemia effects by suppressing other pathways in addition to inhibiting the abnormal BCR signaling.

Our results show that abivertinib can inhibit AML proliferation by inducing apoptosis and cycle arrest in a BTK-independent manner. We demonstrated that abivertinib induced apoptosis by suppressing the

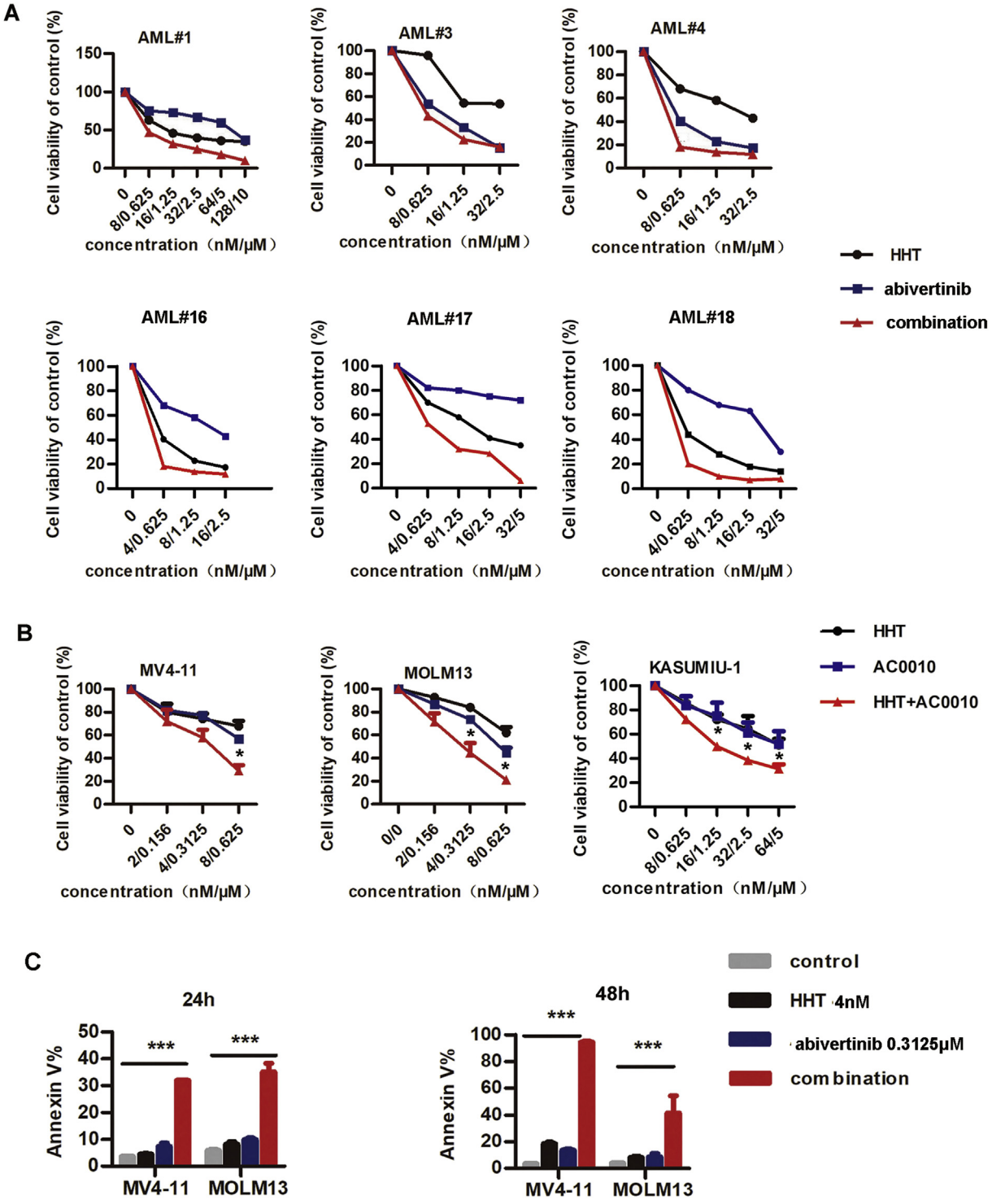

Fig. 5. HHT combined with abivertinib inhibited the proliferation of AML cells A) AML blasts from 6 patients (AML#1 carried FLT3-ITD mutation) were cultured with HHT, abivertinib, or both followed and cell viability analyzed. (B) Reductions in cell viability induced by HHT, abivertinib and HHT and abivertinib combination in MV4-11, MOLM13 and KASUMI-1 cells after incubation of 24 h. (C) Apoptosis induced by HHT, abivertinib, and abivertinib plus HHT after 24 h and 48 h in MV4-11 and MOLM13 cell lines. MV4-11 and MOLM13 cells were treated with DMSO, HHT, abivertinib or HHT plus abivertinib for 24 h. * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$.

anti-apoptosis proteins BCL2 and MCL-1 and activating the caspase family, including caspase-3 and caspase-7. In AML cell lines, abivertinib treatment arrested the cell cycle at G0/G1 phase by downregulating the expression of CDK2, CDK4, and CDK6. Cell cycle arrest is the main mode of action of many antitumor chemotherapy drugs. Cell cycle blockage inhibits DNA synthesis, thereby inhibiting cell proliferation and promoting anti-leukemic effects.

Chiron et al. reported that mantle cell lymphoma (MCL) patients with primary or early-acquired resistance to ibrutinib showed an abnormal activation of the PI3K pathway [26,27]. Ma et al. showed similar results in MCL cell lines [28]. The critical role of PI3K signaling in the progression of numerous tumors, including leukemia, has been well-

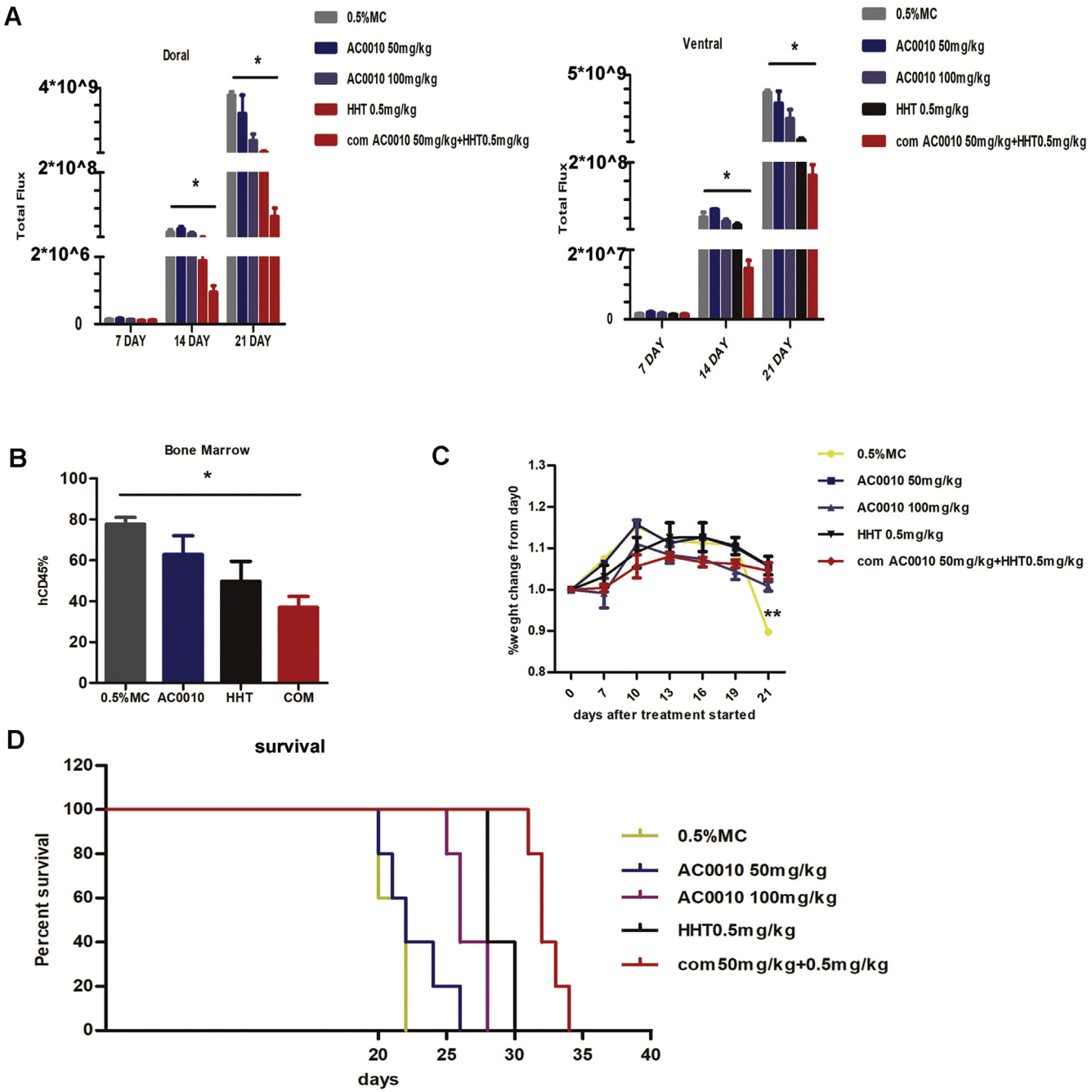

Fig. 6. Anti-leukemia effect of abivertinib plus HHT in MV4-11-lu xenograft mice. (A) Leukemia tumor burden assessed by photon intensity following treatment up to 21 days or sacrifice. (B) Percentages of human-CD45 in the bone marrow of mice from different treatment groups after sacrifice. (C) Body weight change versus time. (D) Kaplan-Meier survival time (time to hemiplegic paralysis) for the duration of the treatment.* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$.

reported [29,30]. Importantly, we show that abivertinib can effectively suppress the expression of PI3K and its downstream signaling proteins, p-AKT, p-IKK, and p-NF-kB, inducing apoptosis in AML cell lines.

On the basis of the obtained results, we hypothesize that abivertinib inhibits AML cell proliferation via BTK-dependent and -independent mechanisms; however, the results do not completely explain the special sensitivity of the cell lines carrying *FLT3-ITD* mutation. It has been reported that ibrutinib affects the STAT5 pathway [21,31,32], which in turn, is closely related to the FLT3 pathway. We also investigated abivertinib sensitivity in cell lines harboring *FLT3-ITD* mutation. Our study indicates that abivertinib also has a significant effect on the FLT3 signaling pathway, by virtue of downregulation of phosphorylation of FLT3 and STAT5, especially p-STAT5. A greater reduction in p-STAT5 levels was observed in cells treated with abivertinib compared with that in cells treated with ibrutinib, suggesting a preferential sensitivity of the cells to abivertinib. These results suggest that an anti-AML mechanism of abivertinib action may offer therapeutic advantages over that of ibrutinib. Abivertinib not only inhibited the hyperactive BTK signaling pathway through a BTK-dependent mechanism but also significantly downregulated the abnormal activation of the PI3K signaling pathway, which might promote resistance to ibrutinib [27,33].

Previously, we have shown that ibrutinib combined with HHT targeted FLT3 and hence achieved a good combination effect in *FLT3-ITD* mutant cell lines [21]. Similarly, in this study, we show that combined treatment with abivertinib and HHT achieved synergistic effects in MV4-11 and MOLM13 cells as well as in KASUMI-1 cells which did not

harbor *FLT3-ITD* mutation. Similar results were also obtained in primary AML blasts. In our study, the combination of abivertinib and HHT showed promising anti-leukemia effects at lower doses of both drugs. It is possible that the low but effective doses of combination therapy observed *in vitro* may offer tolerance advantages *in vivo*, as many elderly and/or physically weak patients are unable to tolerate conventional doses of chemotherapy. Abivertinib plus HHT also induced apoptosis in AML cells, as was detected by both flow cytometry and Western blot analysis. Consequently, our study suggests that the two drugs have a synergistic effect on the PI3K pathway and also in the downstream FLT3 pathway (data not shown), as was shown in our previous study with ibrutinib.

We also investigated the effects of abivertinib *in vivo*. While abivertinib at a lower dose of 50 mg/kg did not significantly prolong the survival time in the MOLM13-SCID and MV4-11-luc-NSG xenograft mouse model, when the dose was increased to 100 mg/kg or combined with HHT, the life span of the mice increased significantly. Photon intensity evaluation revealed that the leukemic burden of mice receiving the combination treatment reduced significantly compared with that in mice treated with individual agents or vehicle ($p < 0.05$). The body weight of treated mice stayed relatively stable when compared with those receiving the vehicle, indicating that the treatment was well-tolerated.

In conclusion, our study indicates that abivertinib inhibited the survival of AML cells, especially those with *FLT3-ITD* mutations, and has a stronger *in vitro* anti-leukemia effect than ibrutinib. In both *in vitro* and *in vivo* experiments, abivertinib combined with HHT was associated with synergistic anti-leukemic effects. We speculate that abivertinib-based treatment regimens may offer the potential to improve the survival of patients with AML. Clinical studies on abivertinib-involved therapy are planned.

Conflicts of interest

The authors declare no conflict of interest.

Ethics approval

Our animal study was approved by Ethics Committee for Laboratory Animals of the First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were exposed to a 10/14 h light-dark cycle, kept under normal room temperature and fed by standard pellet food and tap water.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.07.008.

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