ORIGINAL RESEARCH ARTICLE

Simvastatin Preferentially Targets *FLT3*/ITD Acute Myeloid Leukemia by Inhibiting MEK/ERK and p38-MAPK Signaling Pathways

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Abstract

Background The *FLT3*/ITD mutation exists in many acute myeloid leukemia (AML) patients and is related to the poor prognosis of patients. In this study, we attempted to evaluate the antitumor activity of simvastatin, a member of the statin class of drugs, in vitro and in vivo models of *FLT3*/ITD AML and to identify the potential mechanisms.

Methods Cell Counting Kit-8 (CCK-8) and Annexin V/propidium iodide (PI) staining kits were used to detect cell viability and apoptosis, respectively. Subsequently, Western blot and rescue experiment were applied to explore the potential molecular mechanism. In vivo anti-leukemia activity of simvastatin was evaluated in xenograft mouse models.

Results In vitro experiments revealed that simvastatin inhibited AML progression in a dose- and time-dependent manner, while in vivo experiments showed that simvastatin significantly reduced tumor burden in *FLT3*/ITD xenograft mouse models. After simvastatin treatment of *FLT3*/ITD AML cells, intracellular Rap1 was downregulated and the phosphorylation levels of its downstream targets MEK, ERK and p38 were significantly inhibited. The rescue experiment showed that mevalonate, an intermediate product of the metabolic pathway of mevalonate, and its downstream geranylgeranyl pyrophosphate (GGPP) played a key role in this process. Finally, we demonstrate that simvastatin can induce apoptosis of primary AML cells, while having no effect on peripheral blood mononuclear cells from normal donors.

Conclusions Simvastatin can selectively and effectively eradicate *FLT3*/ITD AML cells in vitro and in vivo, and its mechanism may be related to the disruption of the HMG-CoA reductase pathway and the downregulation of the MEK/ERK and p38-MAPK signaling pathways.

Key Points

This study showed that simvastatin can selectively and effectively eradicate *FLT3*/ITD acute myeloid leukemia (AML) cells in vitro and in vivo.

Simvastatin inhibited the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) on the mevalonate pathway in the *FLT3*/ITD AML cells. Intermediate products of the mevalonate pathway, mevalonate and GGPP, acted as the facilitators, whereby simvastatin exerted its influence.

The antileukemia activity of simvastatin is associated with the disruption of the HMG-CoA reductase pathway and downregulation of the MER/ERK and p38-MAPK signaling pathways.

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1 Background

Acute myeloid leukemia (AML) is a type of clonal malignant proliferative disease of hematopoietic myeloid blasts. The prognosis remains poor in pediatric and adult patients, with 5-year survival rates approximating 66–68% and 10–20%, respectively [1–3]. FMS-like tyrosine kinase 3 (FLT3), a tyrosine kinase type III receptor, is normally present in hematopoietic stem and progenitor cells and plays an important role in early proliferation, differentiation and formation of hematopoietic cells [4]. *FLT3* mutation is one of the most frequently mutated genes in AML, which can lead to constitutive activation of FLT3 in nearly 30% of AML patients [5–7]. The most frequent *FLT3* mutation is internal tandem repeat mutations in the proximal membrane domain, known as *FLT3*-ITD, which happens in about 20–25% of AML patients [8]. *FLT3* mutants can induce the activation of many intracellular signaling pathways, including PI3K/AKT/mTOR, RAS/

MAPK and JAK/STAT5 signaling, which provided a proliferation/survival privilege to cancer cells [9, 10].

Previous studies have confirmed that *FLT3* mutation in AML patients is associated with increased white blood cell count, higher risk of recurrence and poor prognosis, and is one of the important factors for risk stratification [11–13]. Although the introduction of tyrosine kinase inhibitors (TKIs) has yielded positive clinical responses in *FLT3*-mutant AML, several issues that limit the antileukemia activity, e.g. the short-lived partial responses, off-target effects, and drug resistance, as well as the high affordability costs, cannot be neglected [14, 15]. Thus, safe and effective new treatment strategies are sought to achieve better clinical response in patients with *FLT3*-mutated AML.

As we all know, the development of new drugs is a process that requires a lot of time, investment and manpower. Therefore, identifying new indications from existing drugs can greatly shorten the development cycle and reduce the investment, which is a new strategy for drug development. Developed originally as lipid-lowering agents, statins exhibit multiple effects via inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), which is the first rate-controlling step of the mevalonate pathway, responsible for generating low-density lipoprotein receptor (LDLR) feedback to prevent cholesterol formation, supporting plasma cholesterol internalization and disrupting protein prenylation. The increase of mevalonate synthesis is beneficial to the growth of tumor cells. The imbalance of cellular cholesterol homeostasis has been confirmed to protect AML cells from the cytotoxic effect of chemotherapy. The cholesterol levels are increased in AML cells after exposure to chemotherapy drugs, which may reduce the cytotoxicity of these drugs. Therefore, interfering with cholesterol metabolism by using statins to inhibit cholesterol synthesis has been proposed as a method to improve the anti-leukemia treatment strategy [16–20]. Currently, it has been reported that statins exhibit cytotoxicity to a variety of human AML cells, such as in primary CD34 + AML, where the combination of simvastatin and tipifarnib enhances the inhibitory effect on AML cells [21–25]. Moreover, statins also play an important role in *FLT3*/ITD AML. Studies have shown that statins can induce cell death in *FLT3*/ITD AML by inhibiting FLT3 glycosylation, leading to a loss of surface expression [26]. These studies provided a great support for re-positioning statins as anti-leukemia drugs. Several other studies also reported that the use of statins significantly reduced cancer-related risks and prolonged patients' survival rates in gastric, colorectal, and breast cancers [27–29].

In light of the above observations, we sought to evaluate the antitumor activity of simvastatin, a member of the statin class of drugs, in vitro and in vivo models of *FLT3*/ITD AML, and to identify the underlying mechanisms of action. Taken together, these findings suggest that this novel therapy, which reasonably interferes with tumor metabolism, may be a promising option for treating *FLT3*/ITD AML.

2 Material and Methods

2.1 Reagents

Simvastatin was purchased from MedChemExpress (HY-17502; Shanghai, China). Simvastatin 8 mg (20 mM) was dissolved in 0.2 mL absolute ethyl alcohol and 0.25 mL 0.1N NaOH, and was subsequently incubated at 50°C for 2 h and neutralized with hydrochloric acid (HCl) to pH 7.2. Mevalonic acid powder was purchased from MedChemExpress (HY-113071) and was dissolved in dimethylsulfoxide (DMSO) [D12345; Invitrogen, Waltham, MA, USA]. Farnesyl pyrophosphate (FPP) solution was purchased from Sigma-Aldrich (F6892-1VL; St Louis, MO, USA) and geranylgeranyl pyrophosphate (GGPP) solution was purchased from GlpBio (G6025-1VL; Montclair, CA, USA).

2.2 Acute Myeloid Leukemia (AML) Cell Culture Conditions

Human *FLT3*/ITD AML cell lines MOLM-13 and MV4-11, and human stem cell AML cell lines KASUMI and KG-1α were purchased from ATCC (Rockefeller, MD, USA). All cell lines were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, New York, NY, USA) and 1% penicillin/streptomycin. Cells were cultured in a humidified environment of 37 °C and 5% CO_2. All cell lines were mycoplasm-free.

2.3 Primary Samples

This study was authorized by the Ethics Committee of the First Affiliated Hospital of Xiamen University and was implemented according to the guidelines of the Declaration of Helsinki. Informed consent was obtained from all patients. Bone marrow (BM) samples from 10 first-time-diagnosed AML patients, and peripheral blood specimens of 10 healthy hematopoietic stem cell transplantation donors, were collected. Mononuclear cells were isolated from the collected samples by density gradient centrifugation of lymphatic vessels (BD, NJ) and incubated in RPMI 1640 medium supplemented with 10% FBS for subsequent drug trials.

2.4 Cell Viability Assay

The cytotoxicity of simvastatin was determined by Cell Counting Kit-8 (CCK-8) [HY-K0301; MedChemExpress]. First, 2×10^4 cells/well of AML cells were seeded into 96-well plates with a total volume of 100 uL of growth medium and drug in a humidified environment containing 5% CO_2 at 37°C. Second, AML cells were treated with 2.5, 5, 10, 20, or 40 μM simvastatin alone or in combination with 7.5, 15, 30, 60, or 120 μM mevalonic acid, 1.25, 2.5, 5, 10, or 20 μM FPP, or 0.625, 1,25, 2.5, 5, or 10 μM GGPP at different concentrations for 22, 46, or 70 h. Third, 10 μL of CCK-8 reagents were added to each well and incubated in the incubator for an extra 2 h. Finally, absorbance was measured at 490 nm using an enzyme-labeled instrument (ELx800; BioTek Instruments Inc., Winooski, VT, USA). Half of the maximal inhibitory concentration (IC_{50}) was reckoned using Graphpad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

2.5 Cell Apoptosis Assay

2×10^5 cells/well of AML cells were seeded into 12-well plates and treated with 2.5, 5, 10, 20, or 40 μM simvastatin for 24, 48 or 72 h. The cells were then harvested with the supernatant containing floating cells and washed twice with binding buffer. The cells were then incubated with 5 μL Annexin V-FITC for 15 min at 4 °C in the dark, and 5 μL propidium iodide (PI) was added for another 5 min. The excess dye was then washed with ice-cold phosphate-buffered saline (PBS) and followed by flow cytometry (BD Bioscience, Oxford, UK) and Flowjo software (BD Bioscience) analysis.

2.6 Cell Cycle Analysis

First, 2×10^5/well of AML cells were incubated in 12-well plates and exposed to 0, 2.5, 5, or 10 μM simvastatin. After drug exposure for 24 h, cells were harvested, fixed with 70% precooled ethanol, and incubated overnight on ice. The cells were then stained with freshly prepared PI solution for 30 min in the dark (on ice), which contained 200 μg/mL DNase-free RNase A, 20 μg/mL PI, and 0.1% Triton X-100. Finally, cells were subjected to flow cytometry (BD Bioscience) and the cellular DNA complements were analyzed using FACS C6 software.

2.7 Western Blot Analysis

AML cell lines were plated at 2×10^6 cells/well in six-well plates. Cells were treated with 2.5, 10, or 40 μM simvastatin alone or 40 μM simvastatin combined with 120 μM mevalonic acid, 10 μM GGPP, or 20 μM FPP for 48 h. Protein was extracted using the solution method and whole-cell lysates were extracted and quantified from each sample. Reagents used included Ripa Lysate (Beyotime Technology Co. Ltd, Shanghai, China; P0013C), PMSF solution (Beyotime Technology Co. Ltd; ST507-10ml), protease inhibitor (APExBIO Technology LLC, Houston, TX, USA; K1007), phosphatase inhibitor (APExBIO Technology LLC; K1015-A, K1015-B), and BCA protein assay kit (Beyotime Technology Co. Ltd; P0012). After electrophoresis and PVDF membrane transfer, the separated proteins were probed to the primary antibodies at 4°C overnight, then cleaned three times with TBST, and incubated with secondary HRP-conjugated antibodies (1:10,000; Abcam, Cambridge, UK) at room temperature for 1.5 h. The primary antibodies included anti-phospho-ATM (Cell Signaling, #5883S), anti-phospho-ATR (Cell Signaling, #2853S), anti-phospho-CHK1 (Cell Signaling Technology, Inc., Danvers, MA, USA; #2348S), anti-phospho-CHK2 (Cell Signaling Technology, Inc.; #2197S), anti-P53 (Cell Signaling Technology, Inc.; #2527S), anti-p21 (Cell Signaling Technology, Inc.; #2947S), anti-BCL11A (Cell Signaling Technology, Inc.; #75432S), anti-RAS (Cell Signaling Technology, Inc.; #3339S), anti-RhoB (Cell Signaling Technology, Inc.; #63876S), anti-Rap1 (Cell Signaling Technology, Inc.; #2399S), anti-MDR1(Cell Signaling Technology, Inc.; #13342), and antiphospho-MDM2 (Cell Signaling Technology, Inc.; #86934S). The antibody anti-HMGCR (#174830) was purchased from Abcam (UK), and the antibody anti-TBX2 (#0507R) was purchased from Bioss Antibodies (Beijing, China). Blots were visualized using the ECL Western Blotting Detection Kit (GeneFlow, Lichfield, UK).

2.8 Animal Study

Four- to six-week-old BALB/C nude mice (Beijing HFK Bioscience Co. Ltd, Beijing, China) were inoculated subcutaneously with 2×10^6 MOLM-13 cells to construct an *FLT3*/ITD AML mouse model. Three days after inoculation, mice were randomly divided into two groups (five mice per group); the experimental group was intraperitoneally injected with simvastatin 20 mg/kg/day, and the other group was injected with PBS as the control group. The mice body weights were measured daily. After 10 days of abdominal administration, mice were sacrificed and the tumors were resected, weighted, and measured. Tumor volume was reckoned using the formula $V = (L \times W^2)/2$ (*L* indicates length; *W* indicates width). The tumor sections were stained with hematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) to analyze the morphology and death of tumor cells.

2.9 Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) Staining

The fresh tumor tissue blocks of nude mice were fixed in 10% formalin for 24 h. The tissue slides were then prepared and cleaned with clean xylene. After washing with gradient ethanol (100%, 95%, 85%, 75%, 65%), the paraffin slide is then put into the prepared 4% formaldehyde, fixed for 15 min, and washed and digested (20 µg/mL protease K, 8–10 min). The glass slide was incubated in a wet box at 37 °C for 1 h in the dark, and the reaction was then terminated. After further washing, the film was sealed, and, finally, local green fluorescence of apoptotic tissue was detected using an EVOSTMM7000C imaging system fluorescence microscope.

2.10 Statistical Analysis

Mean ± standard error of the mean of at least three independent experiments was used to represent the results. Statistical analyses were carried out using GraphPad Prism 7 software. The two-tailed Student's t test was used to compare the variables between two groups, and one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test were used to compare the variables among multiple groups. A p value <0.05 indicated statistical significance.

3 Results

3.1 Simvastatin Inhibited Cell Proliferation in *FLT3*/ITD AML Cell Lines

CCK8 was used to determine the inhibitory effect of simvastatin on MOLM-13, MV4-11, Kasumi, and KG-1a. The cell viability rates of each cell line at different concentrations of simvastatin for 24, 48 and 72 h are shown in Fig. 1. Exposure to simvastatin resulted in a significant inhibition of cell

viability in the FLT3/ITD AML cells MOLM-13 and MV4-11 in a dose- and time-dependent manner. In contrast, the human non-*FLT3*/ITD AML cell lines KG1a and Kasumi-1 were much less sensitive to simvastatin in the same range of concentrations. The IC_{50} values of MOLM-13, MV4-11, Kasumi, and KG-1a treated with simvastatin for 24, 48 and 72 h were calculated and are summarized in Table 1. The IC_{50} values of KG1A and Kasumi treated with simvastatin for 24 and 48 h could not be calculated; however, their IC_{50} values at 72 h were 77.43 ± 7.42 µM and 58.63 ± 16.54 µM, respectively, while the IC_{50} values of MOLM-13 and MV4-11 at 72 h were only 5.12 ± 0.79 µM and 6.43 ± 2.80 µM, respectively. Overall, these findings indicate that simvastatin suppressed the proliferation of MOLM-13 and MV4-11 cells in a dose- and time-dependent manner, while Kasumi and KG1a cells were more resistant to simvastatin.

3.2 Simvastatin Induced Apoptosis and Cell Cycle Arrest in *FLT3*/ITD AML Cell Lines

Since MOLM-13 and MV4-11 cells were sensitive to simvastatin treatment, flow cytometry was performed to detect the effects of simvastatin regarding cell cycle and apoptosis on these two AML cell lines to further verify the anti-leukemia efficacy of simvastatin. Generally, the apoptosis rate of MOLM-13 and MV4-11 increased correspondingly with

Table 1 IC_{50} values of simvastatin in treating FLT3/ITD-mutant AML cell lines

Cell lines	IC_{50} ± SD (µmol/L)		
	24 h	48 h	72 h
MV-411	43.95 ± 22.72	12.16 ± 3.92	6.43 ± 2.80
MOLM-13	25.30 ± 3.17	18.50 ± 4.78	5.12 ± 0.79
Kasumi	–	–	58.63 ± 16.54
KG1a	–	–	77.43 ± 7.42

IC_{50} half maximal inhibitory concentration, *AML* acute myeloid leukemia, *SD* standard deviation
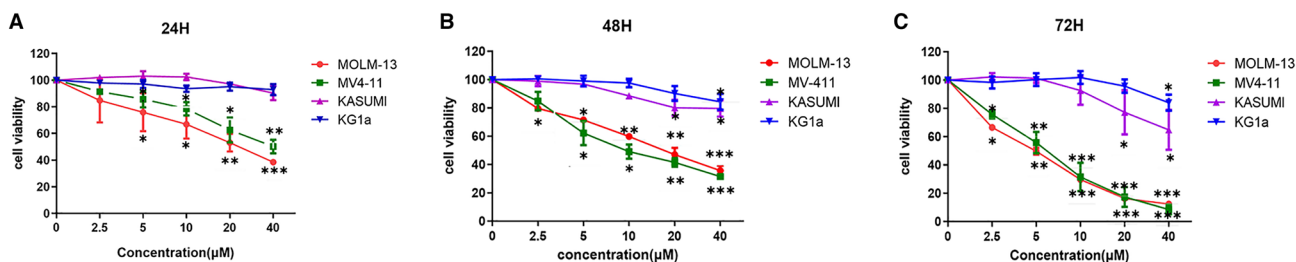

Fig. 1 Effect of simvastatin on AML cell proliferation. MOLM-13, MV4-11, Kasumi, and KG1a were incubated with 0, 2.5, 5, 10, 20, or 40 µM simvastatin for **a** 24 h, **b** 48 h, and **c** 72 h. A CCK-8 kit and Graphpad Prism 7.0 were utilized to analyze cell viability and calculate the IC_{50} value. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus untreated controls. *AML* acute myeloid leukemia, *CCK-8* Cell Counting Kit-8, IC_{50} half maximal inhibitory concentration

the increase in simvastatin concentration and the extension of the incubation time (Fig. 2A–D), of which the tendency was consistent with the results of the cell survival rate. The apoptosis effects of simvastatin on Kasumi and KG-1a were simultaneously tested by using Annexin V/PI double staining. The results showed that there was a statistical difference in the killing effect after 72 h of high concentration (20 and 40 μM for Kasumi, 40 μM for KG1a), while there was almost no effect after 72 h of low concentration or 24 and 48 h of simvastatin treatment (Electronic supplementary Fig. S1a–d). As for the cell cycle shown in Fig. 2E–H, simvastatin induced a G0/G1-phase arrest at a concentration of 10 μM in both MOLM-13 and MV4-11 cells for 24 h, reflected by the increased number of cells in the G0/G1 phase, while cells in the S phase and G2/M were relatively decreased. These data indicated that in addition to inducing cell apoptosis, simvastatin could also play a role by blocking the cell cycle that contributed to its anti-leukemia effect.

3.3 Simvastatin Impaired the Mevalonate, MEK/ERK, and p38-MAPK Pathways in *FLT3*/ITD AML Cell Lines

Simvastatin can suppress tumor cell proliferation and induce cell apoptosis and cell cycle arrest through various mechanisms. Among these, the mevalonate pathway (Fig. 3a) was considered to be the primary mechanism that accounted for the anti-tumor effect of simvastatin. Mevalonate is the precursor of isoprenoids such as FPP and GGPP, and isoprenylation catalyzed by farnesyltransferase or geranylgeranyltransferase is required for small GTPases (e.g. Rho, RAS) to attach to the plasma membrane and subsequently participate in signal transduction pathways that regulate growth and survival [22]. Notably, GGPP produced by the mevalonate cascade also functions as an important substrate in the activation process of Rap1 [30]. Therefore, these effectors of HMGCR were assessed by Western blotting, where we found that the expressions of HMGCR in MOLM-13 and MV4-11 cells were decreased under the action of simvastatin, in concert with the downregulation of Rap1 and RhoB, while the expressions of RAS were upregulated (Fig. 3b). To get an exclusive deep dive, we centered our attention on Rap1-mediated pathways, where we found that the phosphorylation level of MEK, ERK, and p38 were significantly downregulated, which imparted us to speculate that the underlying mechanisms of action of simvastatin relied on the MEK/ERK and p38-MAPK signaling pathways (Fig. 3c, d).

3.4 Mevalonate and Geranylgeranyl Pyrophosphate Can Alleviate the Cytotoxicity Induced by Simvastatin in *FLT3*/ITD AML Cell Lines

To determine which components of the mevalonate pathway are modulated upon simvastatin treatment, we combined simvastatin with three mitigating agents (mevalonate, FPP, GGPP) and tested the rescue effect of each component. The results showed that mevalonate and GGPP could relieve simvastatin from inhibiting AML cell proliferation, while FPP failed to show activity in remission (Fig. 4A, B). Similarly, when the combination of simvastatin plus mevalonate or GGPP was applied, the protein expression of HMGCR and Rap1 in MOLM-13 and MV4-11 cells did not differ significantly from the control group (Fig. 4c, d), which indicates that mevalonate and GGPP functioned as the activators of the mevalonate pathway in accordance with the results of the cell viability assay.

3.5 Simvastatin Reduced Tumor Burden in *FLT3*/ITD Xenograft Mice Models

On the basis of these in vitro results, we were encouraged to detect the anti-leukemia activity of simvastatin in vivo. MOLM-13 cells were used to establish mouse xenograft models by subcutaneous inoculation, the mice were randomly divided into the control and simvastatin treatment groups (Fig. 5a). We observed that although the mice in the simvastatin treatment group had a moderate and short-term weight loss compared with the control group on days 2–4 after drug treatment, they gradually recovered on day 5, and no significant drug toxicity was observed thereafter (Fig. 5b). After 10 days' consecutive administration of simvastatin, the mice were sacrificed and the tumors were harvested and weighed. Compared with the mice in the control group, the tumor size (Fig. 5c) and tumor weight (Fig. 5d) of the mice in the simvastatin treatment group were significantly reduced, as well as tumor volume (Fig. 5e). Immunohistochemistry analysis was also performed and the results showed that the mice in the simvastatin treatment group had fewer infiltrating MOLM-13 cells and more apoptotic cells could be observed (Fig. 5f). In summary, these results indicated that simvastatin may have a certain inhibitory effect on the growth of AML tumors *in vivo*.
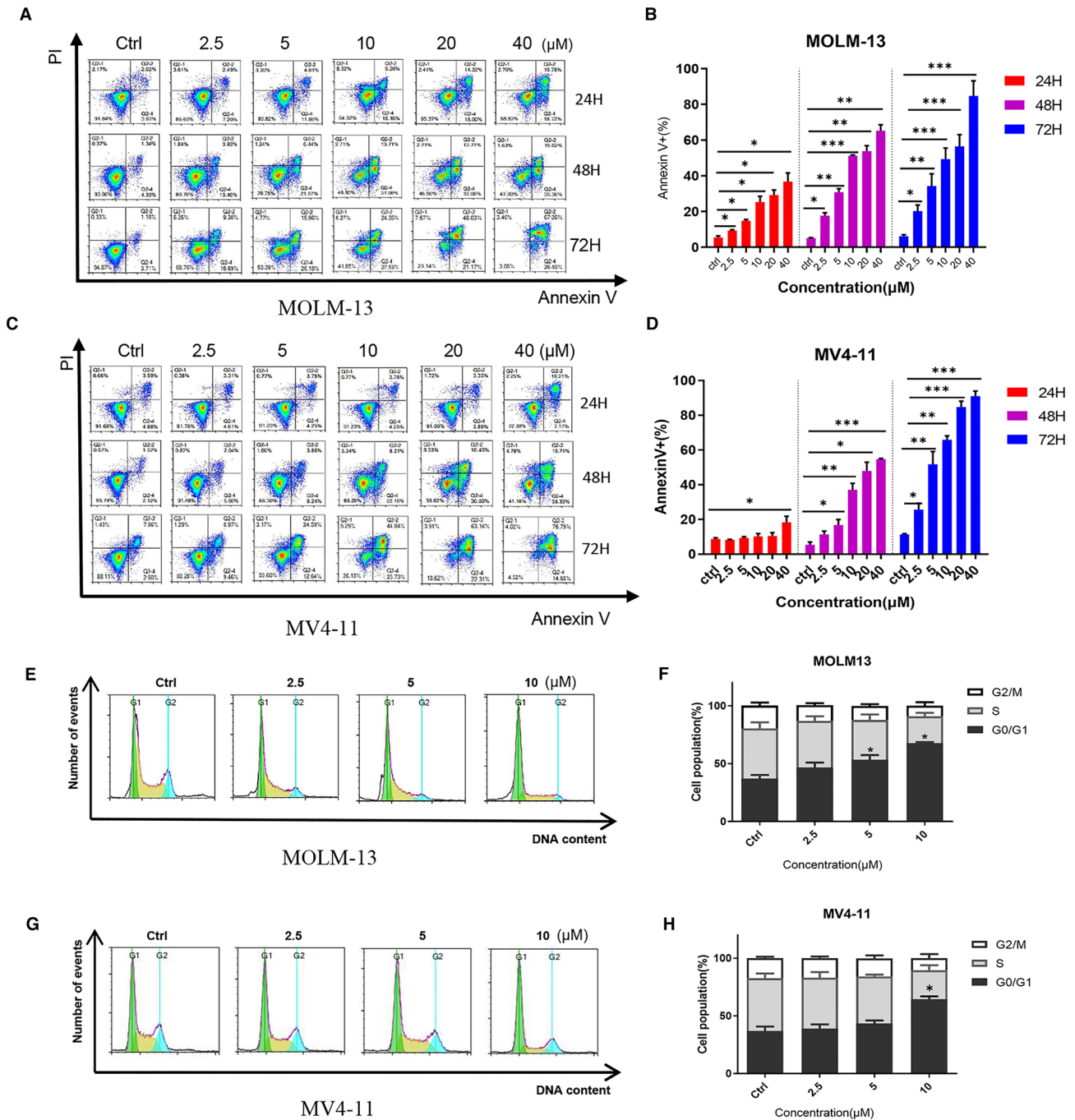
Fig. 2 Simvastatin induced apoptosis as well as cell cycle arrest in *FLT3*/ITD AML cell lines. **a** MOLM-13 and **c** MV4-11 were incubated with the indicated concentrations of simvastatin for 24, 48, and 72 h. Annexin V/PI double staining was then performed to determine the percentage of apoptotic cells. The corresponding numerical statistics of **a** and **c** are displayed in the histograms on the left as **b** and **d**. **e** MOLM-13 and **f** MV4-11 were exposed to 0, 2.5, 5, 10 μM simvastatin for 24 h. PI staining was then performed to analyze the cell cycle ratio of each phase. The corresponding numerical statistics of **a** and **c** are displayed in the histograms on the left as **g** and **h**. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *AML* acute myeloid leukemia, *PI* propidium iodide
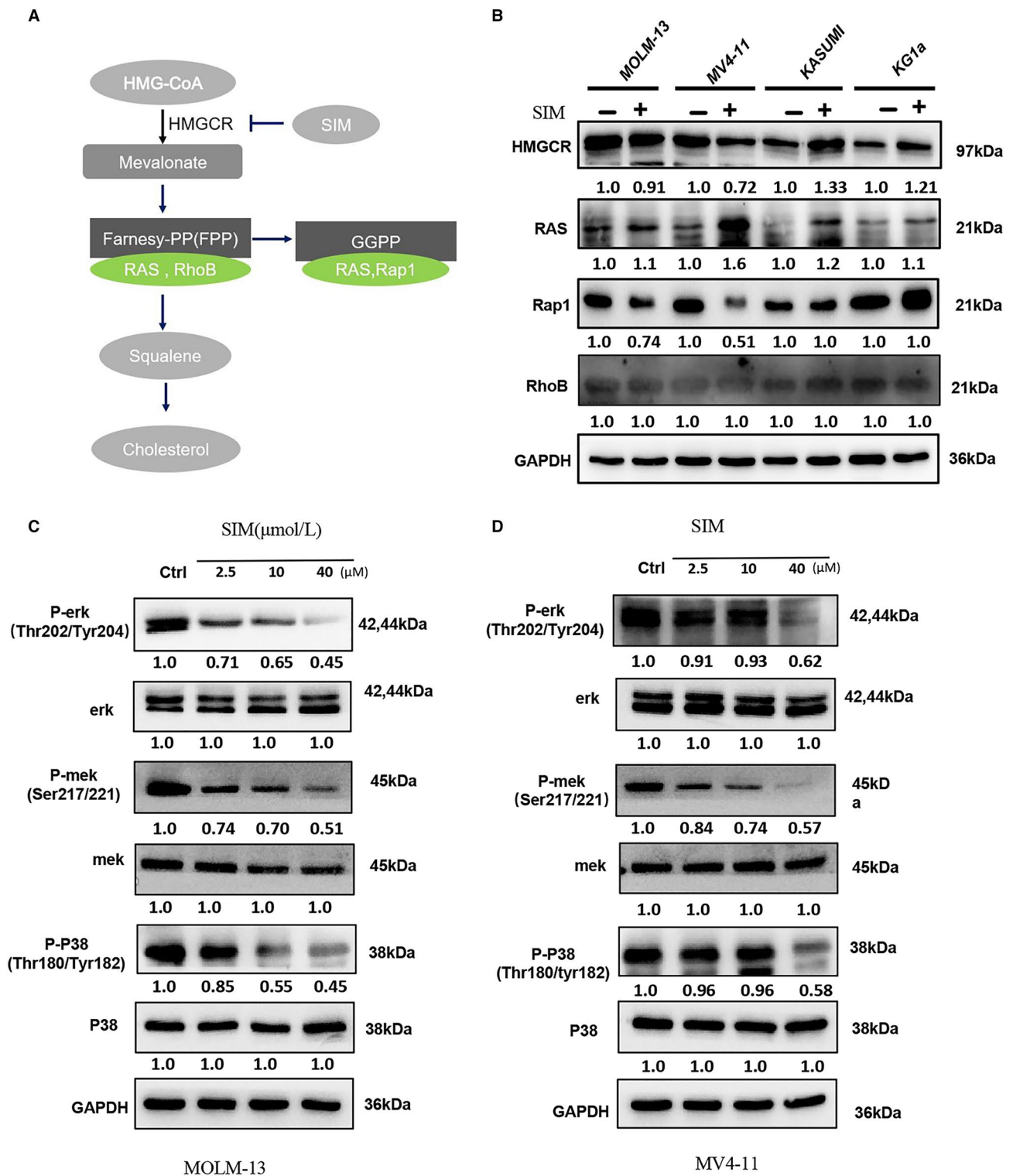
Fig. 3 Effect of simvastatin on the mevalonate pathway and the downstream targets. **a** Illustration of how simvastatin acts on the mevalonate pathway. **b** MOLM-13 and MV4-11 were exposed to 40 μM simvastatin for 48 h, and Western blotting analysis was then performed to determine the protein expression level of HMGCR, RAS, Rap1 and RhoB. **c** MOLM-13 and **d** MV4-11 cells were exposed to the designated concentrations of simvastatin for 48 h, and ERK, MEK and p38 and their phosphorylation levels were detected by Western blotting. *HMGCR* 3-hydroxy-3-methylglutaryl-CoA reductase, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate, *SIM* simvastatin, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase
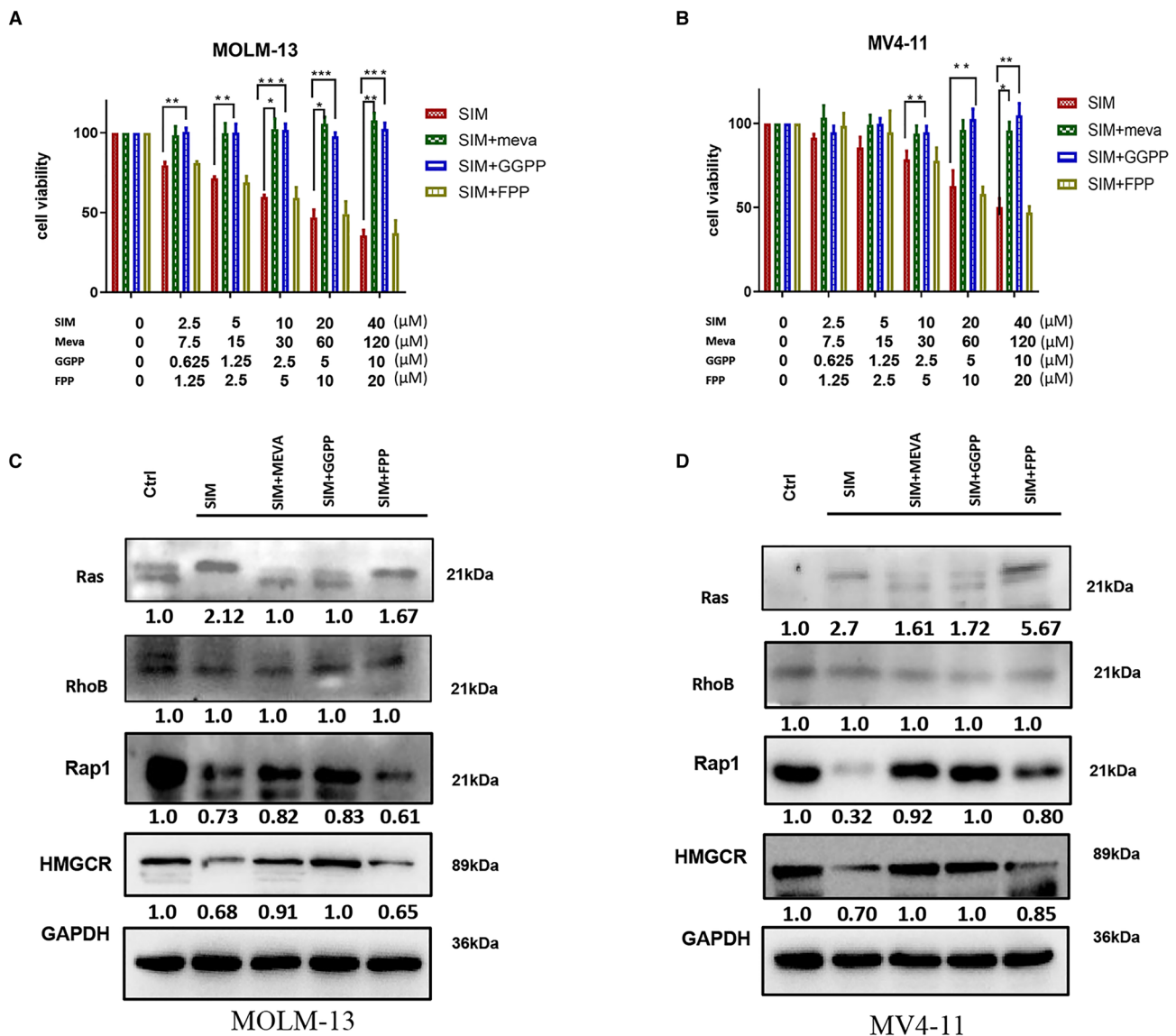
Fig. 4 Mevalonate and GGPP counteracted the actions of simvastatin. **a** MOLM-13 and **b** MV4-11 cells were treated with the indicated concentrations of simvastatin alone or in combination with mevalonate, FPP, or GGPP for 48 h. CCK-8 was used to detect cell viability. The significant groups, with the presence of simvastatin (40 μM) alone or combined with mevalonate (120 μM), GGPP (10 μM) or FPP (20 μM), were then subjected to Western blot analysis to detect the expression level of interested proteins on **c** MOLM-13 and **d** MV4-11 cells. ($p < 0.05$, $p < 0.01$, $p < 0.001$). *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate, *CCK-8* Cell Counting Kit-8, *SIM* simvastatin, *Meva* mevalonate, *Ctrl* control, *HMGCR* 3-hydroxy-3-methylglutaryl-CoA reductase, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

3.6 Simvastatin Attenuated Primary AML Blasts While Sparing Normal Peripheral Blood Mononuclear Cells

Having identified that simvastatin exerts cytotoxicity to AML cells both in vivo and in vitro, we further evaluated the effect of simvastatin on primary AML blasts and normal peripheral blood mononuclear cells (PBMCs). The clinical characteristics of AML patients (including age, sex, white blood cell count, chromosome and immunotype)

are summarized in Table 2. On the one hand, we found that the apoptosis rate of primary cells with FLT3 mutation was higher than that of non-FLT3-mutant primary cells (Fig. 6a). On the other hand, simvastatin exhibited no obvious cytotoxicity to the normal PBMCs under the same condition ($n = 10$) [Fig. 6b]. These results suggested that FLT3-mutant AML blasts were more susceptible to simvastatin-induced growth inhibition than non-FLT3 AML blasts and normal PBMCs.
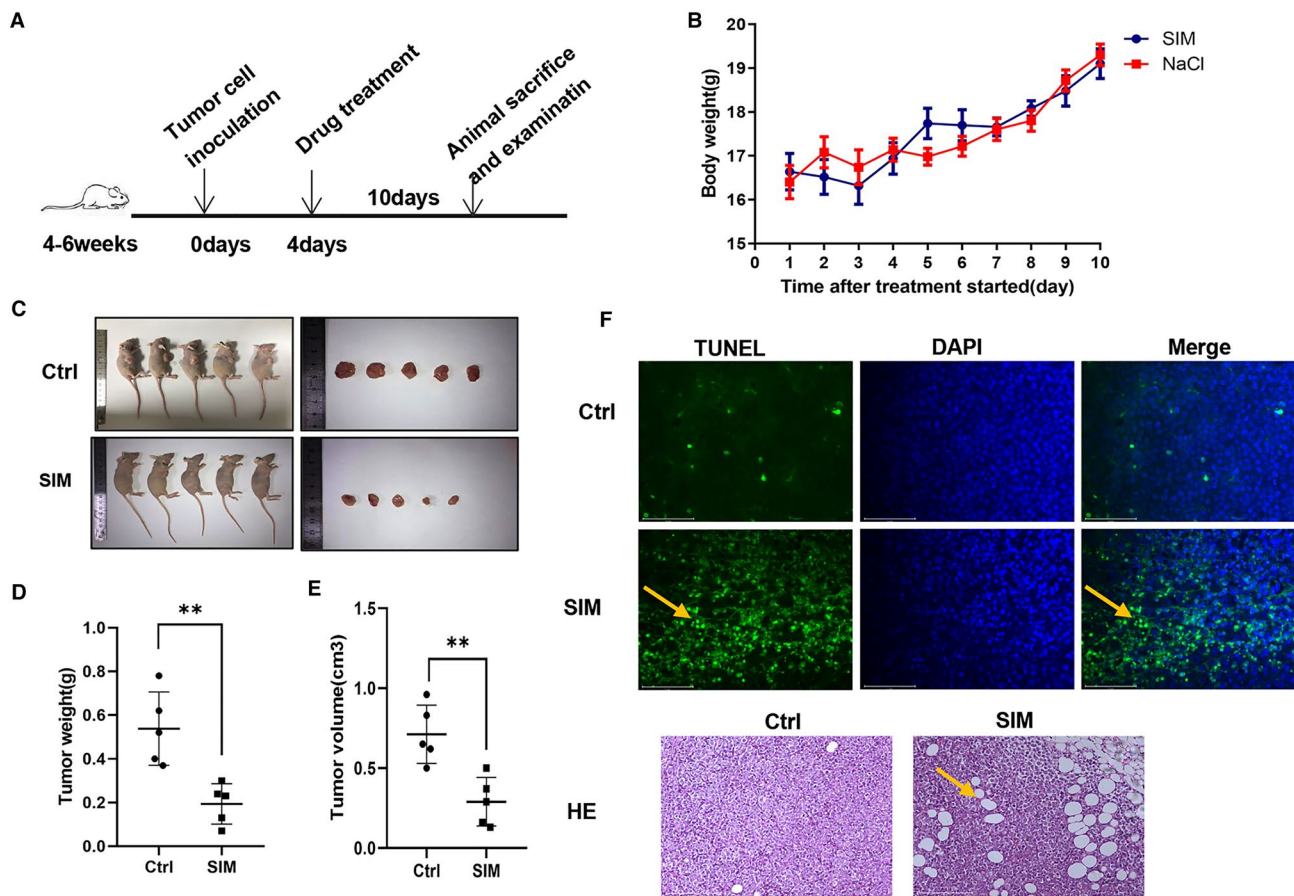
Fig. 5 Simvastatin inhibited tumor growth in *FLT3*/ITD xenograft mice models. **a** Schematic diagram of the animal study procedures (*n* = 5). **b** Mice body weights were measured every day after tumor cell inoculation. **c** After 10 days of continuous drug treatment, the mice were sacrificed and the mice and tumors were then imaged for comparison. The corresponding numerical statistics of **d** tumor weight and **e** tumor volume were also determined. The tumor tis-

sue sections were stained with H&E and TUNEL for immunohistochemistry analysis. **f** Cells with green fluorescence were positive for TUNEL staining, and apoptosis occurred. H&E staining: inflammatory cells infiltrated and vacuoles increased in the medication group. ***p* < 0.01. *H&E* hematoxylin and eosin, *TUNEL* terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling, *Ctrl* control, *SIM* simvastatin, *DAPI* 4′,6-diamidino-2-phenylindole

4 Discussion

FLT3/ITD mutation serves as an important indicator of AML regarding diagnosis, prognosis and relapse, which would help to guide the therapeutics decisions throughout a patient's disease course [31]. Since 2006, targeting FLT3 in AML with TKIs had been granted marketing authorization and has achieved encouraging results. However, the defects of TKIs, like the elevation of FLT3 ligand levels in AML patients, which could compensate for the outcome, are severe and hard to tackle, thus calling for new remedies to overcome some of the challenges. Since metabolic reprogramming is a hallmark of malignancy, targeting metabolic reprogramming has emerged as an important strategy for anticancer therapy [32]. Like abnormal glucose and glutamine metabolism, abnormal cholesterol metabolism also plays a key role in tumors. Cholesterol

metabolism is closely related to tumor genesis, development, and prognosis. Abnormal changes in genes related to cholesterol metabolism enzymes have been shown to affect tumor cell proliferation, invasion and migration in prostate, breast, colorectal and brain cancers [33]. As the rate-limiting enzyme for cholesterol biosynthesis, HMGCR has been identified as a potential target for the therapy of cancers. Currently, the repositioning of statins in the treatment of cancer has attracted a lot of attention. Statins have been developed and are widely used as a serum cholesterol-reducing drug that functions as an HMGCR inhibitor by preventing the conversion of HMG-CoA into mevalonate and inhibiting cholesterol synthesis [34, 35]. Since malignant cells are prone to elevating mevalonate synthesis, which promotes the growth of tumor cells [17, 18], HMGCR is thus equipped with antiproliferation activity, at least in part, by inhibiting the formation of mevalonate.

Table 2 Clinical characteristics of AML patients ($n = 10$)

Patient no	Age (years)	Gender	FAB subtype	Cell count ($\times 10^9$/L)				Karyotype	Molecular features	Induction therapy	Status
				WBC	HB	PLT	Blast				
1	63	F	M2	31.7	61	159	84	47,XX,+4[12]/46,XX[6]	NPM1, FLT3,TET2,SH2B3	IA	CR
2	39	F	M5	70.59	45	40	91	46,XX[20]	DNMT3A,IDH1,NPM1	IA	CR
3	39	M	M2	14.25	89	21	67.5	46,XY,del(9)(q12q22)[12]/46,XY[8]	CEBPA	IA	CR
4	28	M	M5	106	74	126	91	46,XY[7]	WT1,KRAS-K117R	IA	CR
5	80	M	M0	68.68	63	53	51.5	–	–	–	–
6	68	M	M5	74.69	63	75	13.5	47,XY,+mari(21q)/46,XY	NPM1,TET2	IAA	NR
7	66	F	M0	90.3	115	37	93.5	46,XX[20]	WT1,FLT3-ITD,NPM1,TET2	HIA	CR
8	65	M	M2	106.18	110	19	98	46,XY[20]	WT1,FLT3-ITD,NPM1,TET2	IA	CR
9	75	M	M5	14.48	81	31	37	46,XY[20]	ASXL1,DNMT3A,FLT3,IDH2,NPM1	IA	NR
10	35	M	U	3.52	94	62	41	46,XY[2]	TP53	HSCT	CR

AML acute myeloid leukemia, *F* female, *M* male, *WBC* white blood cells, *HB* hemoglobin, *PLT* platelet, *CR* complete response, *NR* no response, *HSCT* hematopoietic stem cell transplantation

Additionally, inhibition of HMG-CoA reductase can also reduce the production of other intermediate products of the mevalonate pathway, including isoprenylation, FPP and GGPP [36], which suggests that statins could have pleiotropic mevalonate-independent effects via these intermediate products. In this study, we confirmed that simvastatin inhibited the HMGCR on the mevalonate pathway in the *FLT3*/ITD AML cells (Fig. 3b). Moreover, the rescue experiments (Fig. 3c, d) further consolidated that intermediate products of the mevalonate pathway, mevalonate and GGPP, but not FPP, acted as the facilitators whereby simvastatin exerted its influence. This is because a second molecule, isopentenyl PPi, is required to convert FPP to GGPP. As isopentenyl PPi is also depleted by statin exposure, it will not be utilized by statin-treated cells [37]. A previous study showed that statins could inhibit the glycosylation of FLT3, which led to the loss of surface expression and induced cell death [26]. The difference is that our research starts with another lipid metabolic pathway, showing that statins can inhibit the synthesis of hydroxymethylglutaryl-coenzyme A reductase (HMGR) through another lipid metabolic pathway, reduced synthesis of its downstream GGPP, which undoubtedly enriched the mechanistic network of statin therapy for *FLT3*/ITD + AML.

Notably, the GGPP produced by the mevalonate cascade also functions as an important substrate in the activation process of Rap1 [30], which is involved in many biological processes, including cell adhesion, cell growth, cell apoptosis, cytoskeleton remodeling, and intracellular vesicle transport [38, 39]. In adrenal tumors and ovarian tumors, Rap1 can participate in the proliferation and migration process by activating the ERK/MAPK, MER3/6/p38-MAPK, and PI3K/AKT pathways [40, 41]. Rap1 and RAS have high sequence similarity, and they have overlapping binding partners. Rap1 has also been shown to oppose and mimic RAS-driven cancer phenotypes. RAS and Rap1 cooperate to initiate and maintain ERK signal transduction, which is activated in many malignant tumors [42]. In our study, we found that expression of Rap1 was downregulated and expression of RAS was upregulated after simvastatin treatment. We hypothesized that this could be due to the fact that Rap1 and RAS have overlapping binding partners; simvastatin-induced downregulation of Rap1 resulted in relative upregulation of RAS, but this did not affect the inhibition of downstream ERK signaling (Fig. 3c, d). At the same time, although RAS has always been considered an oncogene that promotes cell proliferation, we clearly observed inhibition of AML cell proliferation after the use of simvastatin (Fig. 1). Intriguingly, FLT3 is the upstream receptor of the RAS/RAF/MER/ERK signaling cascade, which links it to one of the explanations for the underlying mechanism of simvastatin in the *FLT3*-mutant AML cells.
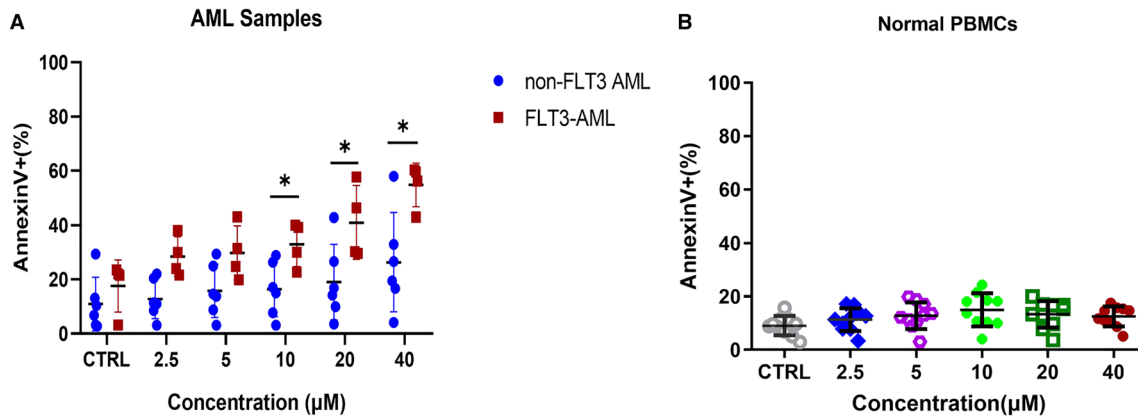
Fig. 6 Effect of simvastatin on primary AML blasts and normal PBMCs. **a** Apoptosis ratios of primary AML bone marrow mononuclear cells and **b** healthy human PBMCs after treatment for 48 h by the indicated concentrations of simvastatin. *$p < 0.05$. *AML* acute myeloid leukemia, *PBMCs* peripheral blood mononuclear cells, *Ctrl* control

5 Conclusions

The present study demonstrates that simvastatin selectively and effectively eradicates *FLT3*/ITD AML cells both in vitro and in vivo. Mechanistically, the anti-leukemia activity of simvastatin is associated with the disruption of the HMG-CoA reductase pathway and downregulation of the MER/ERK and p38-MAPK signaling pathways. Together, this preclinical study creates such high expectations and merits future studies to optimize *FLT3*/ITD AML regimens by combining simvastatin with TKIs and other traditional chemotherapy drugs.

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Declarations

Conflict of interest Genhong Li, Jingwei Yao[2], Zhen Lu, Lian Yu, Qinwei Chen, Lihong Ding, Zhihong Fang, Yin Li, and Bing Xu declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics approval and consent to participate This human tissue samples experiment was approved by the First Affiliated Hospital of Xiamen University Ethics Review Board in accordance with the Declaration of Helsinki. Informed consent was obtained from each participant. All xenograft mouse experimental protocols were approved by the Animal Care and Use Committee of Xiamen University.

Availability of data and materials The datasets and materials in the current study are available from the corresponding author upon reasonable request.

Authors' contributions XB, YJW, LY and FZH designed the study, analyzed the data, wrote the paper and supported the experiments. LGH and LZ conceptualized and designed the study, performed the CCK8, flow cytometric analysis and Western blot experiments as well as analyzed the data. YL, CQW and DLH performed animal experiments. All authors read and approved the final manuscript.

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