

# Targeting of STAT5 using the small molecule topotecan hydrochloride suppresses acute myeloid leukemia progression

JIAHUI LI<sup>1\*</sup>, BIN TANG<sup>2\*</sup>, YING MIAO<sup>3</sup>, GUIHONG LI<sup>4</sup> and ZHENLIANG SUN<sup>1</sup>

<sup>1</sup>Fengxian Hospital Affiliated to Anhui University of Science and Technology, Shanghai 201499; <sup>2</sup>Department of Gynecology, East China Normal University Wuhu Affiliated Hospital (The Second People's Hospital of Wuhu City), Wuhu, Anhui 241000;

<sup>3</sup>East China Normal University and Shanghai Fengxian District Central Hospital Joint Center for Translational Medicine, Shanghai Key Laboratory of Regulatory Biology Institute of Biomedical Sciences and School of Life Sciences,

East China Normal University, Shanghai 201100; <sup>4</sup>Fengxian Hospital Affiliated to The Southern Medical University, Shanghai 201499, P.R. China

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**Abstract.** Acute myeloid leukemia (AML) is a common type of acute leukemia in adults and relapse is one of the main reasons for treatment failure. FLT3-ITD mutations are associated with poor prognosis, short disease-free progression survival and high relapse rates in patients with AML. STAT5 is activated by FLT3-ITD and drives the pathogenesis of AML. STAT5 activation is usually a hallmark of hematologic malignancies and occurs in ~70% of patients with AML. Moreover, STAT5 is a key molecule which regulates hematopoiesis, and its high expression is closely associated with drug resistance, thus direct targeting of STAT5 for AML is of great clinical value. The present study introduces a new small-molecule inhibitor that targets STAT5, presenting a promising approach for AML therapy. A high throughput fluorescence polarization (FP) screening system for STAT5 was designed and established, and used to screen an existing compound library to obtain the highly active small molecule inhibitor, topotecan hydrochloride. Topotecan hydrochloride was demonstrated to be an effective inhibitor of STAT5 by molecular docking prediction and cellular thermal shift assay. Topotecan hydrochloride bound to STAT5, inhibiting its dimerization, phosphorylation and transcription of specific target genes. The compound exhibits cellular activity at the nanomolar level and significantly inhibits the proliferation of human AML cell lines and FLT3-ITD<sup>+</sup> AML cells. Furthermore, topotecan hydrochloride

has the potential to exert an anti-tumor effect *in vivo*. Overall, topotecan hydrochloride offers a new opportunity for the treatment of AML and other hematologic malignancies by directly targeting STAT5.

## Introduction

Acute myeloid leukemia (AML) is a common hematopoietic malignancy that mainly affects adults (1), with the incidence increasing with age (2). AML was reported to be responsible for 11,540 deaths in the United States in 2022, accounting for ~48% of leukemia-related deaths (3). Despite improvements in survival rates for younger patients with AML, due to advances in intensive chemotherapy regimens and supportive care, survival rates for older adults have not significantly improved in the past decade (4,5). The prognosis for AML patients remains poor (6), with 5-year relative survival rates of 68% for patients under 20 years of age and only 8.2% for patients over 65 years of age (3). Recurrence of leukemia is one of the main causes of treatment failure.

Signal transducer and activator of transcription 5 (STAT5) was initially identified as a PRL-activated sheep mammary gland factor (7). There are two predominant isoforms of STAT5, STAT5a and STAT5b, with the former being more prevalent in the mammary gland and the latter in the liver. The two transcription factors are >90% homologous at the protein level, with differences mainly located in the SH2 domain and transactivation domains required for activation. A previous study reported that STAT5a and STAT5b have different functions in hematopoietic stem cells (HSC) and leukemia stem cells (LSCs), and that STAT5b is the driving force behind the maintenance and self-renewal of these cells (8). In the hematopoietic environment, pluripotent hemopoietic stem cells undergo continuous self-renewal, lineage commitment and terminal differentiation to generate a sufficient number of fully developed hematopoietic cells. The expression of STAT5 is crucial for the maintenance and expansion of human stem/progenitor cells during normal and leukemic hematopoiesis. STAT5 expression regulates the self-renewal

*Correspondence to:* Professor Zhenliang Sun, Fengxian Hospital Affiliated to Anhui University of Science and Technology, 6600 Nanfeng Road, Fengxian, Shanghai 201499, P.R. China  
E-mail: zhenliang6@126.com

\*Contributed equally

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and differentiation of human stem/progenitor cells, as well as the survival and proliferation of mature blood cells. As LSCs can self-renew and cause the recurrence of hematopoietic malignancies, their elimination is the primary therapeutic goal. Furthermore, hematopoietic malignancies often exhibit enhanced STAT5 signaling, which is usually due to STAT5b function acquisition via gain-of-function mutations (GOF) or activation by upstream oncogenic kinases (9-15).

STAT5 is an important transcription factor at the hematopoietic level, and is involved in the self-renewal, proliferation and apoptosis of numerous cytokines (16-18). STAT5 acts as both a tumor suppressor and an oncogene that drives disease progression in AML and other cancers. STAT5 signaling dysregulation is frequently caused by constitutive activation due to overexpression, increased receptor signaling or loss of negative regulatory factors (19). This dysregulation is commonly associated with the occurrence and progression of hematologic malignancies, including AML, where ~70% of patients show STAT5 activation in AML cells (20). STAT5 activation is a major driver in the development and progression of certain types of cancer, including AML and non-small cell lung cancer, which are associated with high mortality rates (21-23). Therefore, there is an urgent need to develop new therapeutic strategies that inhibit STAT5 activation.

In AML, FMS related tyrosine kinase 3 internal tandem duplication (FLT3-ITD) mutations constitutively activate the FLT3 receptor, produce abnormal STAT5 signaling expression and drive cell survival and proliferation. Therefore, understanding the mechanism of STAT5 activation and expression could help to develop new therapeutic strategies for STAT5-activated cancers, including FLT3-ITD<sup>+</sup> AML. For all patients with AML, ~30% are affected by FLT3 gene mutations and ~25% have FLT3-ITDs (24). Furthermore, the JAK2-STAT5 signaling pathway is the main downstream signaling pathway of FLT3. Elevated STAT5 phosphorylation levels (25,26) in samples from patients with AML have been previously reported to activate FLT3 mutations, which leads to constitutive activation of STAT5 signaling in AML (27). Therefore, STAT5 maintains constitutive phosphorylation in FLT3-ITD<sup>+</sup> AML cells in the FLT3 signaling pathway (28). FLT3-ITD is a negative prognostic marker for AML (29,30).

Although FLT3 inhibitors have shown clinical efficacy, chemotherapy resistance resulting from STAT5 activation mutations remains a significant obstacle in the treatment of FLT3-ITD<sup>+</sup> AML (31). Consequently, treatment outcomes for these patients are often unsatisfactory. Recent progress in biomolecular drug targets provides a promising approach to improve the survival rate of FLT3-ITD leukemia patients. One potential alternative strategy is to directly target STAT5. Targeting STAT5 is a promising treatment approach for AML and other hematological malignancies, such as AC-4-130 (32).

When cytokines or growth factors bind to receptors, JAK is phosphorylated and activated, which leads to the phosphorylation of downstream target proteins. Transcription factor STAT is then recruited and phosphorylated, forming a dimer that enters the nucleus and binds to target genes (33). This process regulates downstream gene transcription and affects cell proliferation, differentiation and apoptosis. Phosphorylated tyrosine residues serve as binding sites for the SH2 domain in STATs. Homologous or heterodimer formation occurs through

phosphorylated tyrosine-SH2 interaction and are immediately transferred to the nucleus. Direct inhibition of STAT5 can be achieved by disrupting tyrosine phosphorylation, dimerization, DNA binding and nuclear translocation. As dimerization is a critical step in the regulation of STAT5 function, blocking this process is the most effective strategy for directly inhibiting abnormal STAT5 signaling in hematopoietic cancers (34,35). Therefore, the main focus of designing and identifying selective STAT5 inhibitors is to interrupt the formation of dimers by inhibiting STAT5 phosphorylation (36).

In the present study, a novel small-molecule inhibitor targeting STAT5, topotecan hydrochloride (laboratory compound library no. 108), was identified by screening the laboratory compound library.

## Materials and methods

**Cell culture.** All cells used in the present study were supplied by the Zhengfang Yi laboratory at East China Normal University. THP-1 and OCI-AML3 cells were cultured in 80% RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 20% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. MOLM13, HL60, NB4, PBMC and KG-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. Human umbilical vein endothelial cells [HUVECs; cat. no. iCell-h110; 5th passage; Saibaikang (Xiamen) Biotechnology Co., Ltd.] were cultured in primary endothelial cell basal medium [cat. no. PriMed-iCell-002, Saibaikang (Xiamen) Biotechnology Co., Ltd.].

**Reverse transcription-quantitative (RT-q)PCR.** RNA extraction from MOLM13 and KG1 cells was performed using Trizol (Takara Bio, Inc.). cDNA synthesis was performed using a PrimeScript model RT kit (Takara Bio, Inc.) according to the manufacturer's methods. Thermocycling for RT-PCR was as follows: 37°C for 30 min, 85°C for 5 sec, 16°C for 1 h. Thermocycling for qPCR was as follows: 95°C for 10 min; 40 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec and 95°C for 5 min; then 72°C for 6 min and 16°C for 5 min. RT-qPCR was performed using three technical replicates on a QuantStudio3<sup>®</sup> platform (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Premix Ex Taq (Takara Bio, Inc.) and normalized using the 2<sup>-ΔΔC<sub>q</sub></sup> method (37) to calculate mRNA expression levels. β-actin was used for normalization. Primer sequences (Genewiz, Inc.) used were as follows: β-actin forward (F), GTACGCCAACACAGTGCTG and reverse (R), CGTCATACTCCTGCTTGCTG; cellular-myelocytomatosis viral oncogene (CMYC) F, GTCAAGAGGCGA ACACACAAC and R, TTGGACGGACAGGATGTATGC; and STAT5B F, GAGGTGCGGCATTATTTATCCC and R, GCGGTCATACGTGTTCTGGAG.

**Cell cycle analyses.** Cells were treated with 0, 10, 30 or 100 nM topotecan hydrochloride at 37°C for 24 h, cell suspensions were centrifuged at 1,500 x g at 4°C for 3 min, then re-suspended with pre-cooled PBS, centrifuged again and re-suspended in pre-cooled 75% ethanol and immobilized at 4°C overnight. The immobilized cells were centrifuged at

1,500 x g at 4°C for 3 min then re-suspended with pre-cooled PBS, centrifuged again and re-suspended in 1  $\mu$ l RNase (cat. no. EN0601; Thermo Fisher Scientific, Inc.) and 5  $\mu$ l propyl iodide (MilliporeSigma) was added to each PCR tube and stained for 30 min 4°C. Transferred to flow tubes and analyzed using a FACSCanto II (BD Diagnostics) flow cytometer and FlowJo (version, 7.6; BD Diagnostics).

**Apoptosis analysis.** MOLM13 and KG1 cells were seeded at a density of  $3.3 \times 10^6$  cells per 6 cm dish and treated with either DMSO or a range of concentrations of topotecan hydrochloride at 37°C for 2 days. Cell cycle staining was performed using the Cell Death Detection® kit (Nanjing KeyGen Biotech Co., Ltd.) and membrane junction V-FITC Detection® kit (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's protocol. After staining, the cells were washed three times with pre-cooled PBS. The cells were re-suspended in 1x binding buffer from the V-FITC detection kit. The suspension was transferred to flow tubes and analyzed using a FACSCanto II (BD Diagnostics) flow cytometer and FlowJo (version, 7.6; BD Diagnostics).

**Fluorescence polarization (FP) assay.** FP assays were performed according to a previously described protocol (38). The stability of the system was tested, and the optimum culture temperature, time and strength were determined. The effect of DMSO on the system was assessed to be negligible. Briefly, total protein content was calculated and the protein concentration was diluted to 200 nM with FP buffer. A total of 8  $\mu$ l FP buffer (1 mM HEPES, pH 7.5, 5 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT), 200 nM His-STAT protein diluent (STAT1-6) and 2  $\mu$ l compound diluent was added to each well and incubated for 60 min at 37°C. Subsequently, 10 nM labeled peptide diluent was added to each well, away from light. After incubation for 1 h at room temperature, measurements were performed using a Cytation5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Inc.). The generated data was analyzed using GraphPad Prism 7.0 (Dotmatics). The compound library (cat. no. L2110; TargetMol Chemicals, Inc.) was then screened.

**Molecular docking.** The molecular docking of topotecan hydrochloride with STAT5-SH2 domain protein was constructed using AutoDock Vina 1.2.2 software (<https://vina.scripps.edu/>).

**Cellular thermal shift assay (CETSA).** MOLM13 cells ( $3.3 \times 10^6$  cells/dish) were plated and treated with medium containing DMSO or 10  $\mu$ M topotecan hydrochloride at 37°C for 1 h. Cells were collected, washed with PBS three times, and then re-suspended with 1 ml PBS supplemented with protease inhibitors, phosphatase inhibitors and PMSF (10  $\mu$ l each). The cell suspensions were transferred to PCR tubes and heated at 40.0, 40.8, 42.2, 44.6, 47.4 or 49.5°C for 3 min using a PCR instrument. After heating, the cells were transferred to PCR tubes, freeze-thawed with liquid nitrogen for two rounds, and centrifuged at  $6,000 \times g$  at 4°C for 20 min. The lysates were diluted with 5x loading buffer and the protein samples were boiled at 100°C for 15 min. Then the samples were assessed using western blotting.

**Cell proliferation assay.** Cell proliferation assays were performed at 37°C. AML cells were inoculated into 96-well plates with a density of  $5 \times 10^3$  cells per well. HUVEC and PBMC cells were inoculated with a density of  $1 \times 10^4$  cells per well. The cells were incubated overnight in a 37°C, 5% CO<sub>2</sub> incubator, observed to assess adhesion and treated with 1  $\mu$ M topotecan hydrochloride, 50  $\mu$ M AC-4-130 or 30  $\mu$ M Pimozide after cell adhesion, for 72 h. A total of 20  $\mu$ l MTS was added to each well away from light and incubated in a 37°C incubator for 20-50 min. The optical density (OD) of each well was measured at 490 nm using an Spectra Max 190 enzyme spectrometer (Molecular Devices LLC), and the OD of all samples were recorded when the OD of the control reached 0.8-1.0.

**Western blotting.** Western blotting was performed according to a previously reported method (39). Briefly, MOLM13, KG1 and NB4 cells were treated with topotecan hydrochloride for 24 h, the cells were then collected and the protein was extracted using RIPA (cat. no. P0013B; Beyotime Institute of Biotechnology). The protein content was determined using the BCA method and the protein samples were adjusted to 100  $\mu$ g/30  $\mu$ l. A total of 100  $\mu$ g/lane protein was loaded onto 10% SDS-PAGE gels. The blots were transferred to nitrocellulose membranes which were blocked with 5% skim milk solution for 1 h at room temperature. The membranes were incubated with antibodies against STAT5 (1;500; cat. no. 25656T; Cell Signaling Technology, Inc.), phosphorylated (p)-STAT5<sup>Y694</sup> (1;500; cat. no. 4322T; Cell Signaling Technology, Inc.), CMYC (1:1,000; cat. no. ab32072; Abcam) and GAPDH (1:1,000; cat. no. ab181602; Abcam) overnight at 4°C. IRDye 680/800 (both 1:10,000; cat. nos. 926-32221 and 926-32210; LI-COR Biosciences) were used as the secondary antibodies and samples were incubated with these antibodies for 1 h at room temperature.

**IL-3 and GM-CSF stimulating factor Western blotting.** MOLM13 cells were cultured in medium without fetal bovine serum and starved for 24 h (GM-CSF) or 48 h (IL-3). Different concentrations of IL-3 and GM-CSF were added to stimulate the cells for 20 min. Then the cells were collected and western blotting was performed, according to the aforementioned method, to assess protein expression levels. Cells were starved for 24 or 48 h in medium without fetal bovine serum and then treated with a range of concentrations of topotecan hydrochloride for 24 h. Cells were then stimulated with 5 ng/ml IL-3 and GM-CSF for 20 min and subjected to western blotting, according to the aforementioned method.

**Subcutaneous AML xenograft model.** Male NOD/SCID mice (n=20; 4-6 weeks) were purchased from Jiangsu Huachuang Xinnuo Pharmaceutical Technology Co., Ltd. (animal licence no. SCXK2020-0009) and raised in a sterile environment. Mice were housed in a 12 h light/dark cycle at 20-26°C and 40-70% relative humidity with *ad libitum* food and water. MOLM13 cells ( $5 \times 10^6$ ) were suspended in PBS containing 20% Matrigel and injected into the underarm of the right forelimb of the mice. Tumors grew to a volume of 150-250 mm<sup>3</sup>, and the mice were then randomly divided into groups (n=5) as follows: i) Control, ii) 1 mg/kg/day topotecan hydrochloride

via gavage, iii) 3 mg/kg/d topotecan hydrochloride via gavage and iv) positive control (3 mg/kg/day Azactidine via intraperitoneal injection at weeks 1, 3 and 4, 5 days a week). Body weight and tumor volume were measured after 4 days, with the volume calculated using the formula,  $\text{volume} = \text{length} \times \text{width}^2/2$ . Mice were sacrificed by cervical dislocation when the tumor reached a volume of 2,000 mm<sup>3</sup> and administration of the treatment continued until mice were sacrificed. Solid tumors were removed for later western blotting assay and immunohistochemical analysis. The heart, liver, spleen, lung and kidney tissues of one mouse in each group were collected for hematoxylin and eosin (H&E) staining analysis.

***In situ AML model.*** MOLM13 cells (1x10<sup>6</sup>) which were purchased already stably expressing luciferase (MOLM13-Luc; cat. no. NM-B28-1; Shanghai Model organisms) were injected into the tail veins of NOD/SCID mice pre-irradiated with 2.5 Gy. After 4-5 days, the mice were subjected to bioluminescence imaging using an IVIS Lumina III Small animal live optical two-dimensional imaging system and then divided into four groups, with animals evenly distributed based on tumor sizes as indicated by the luciferase luminescence values. The mice were treated as follows: i) Control, ii) 1 mg/kg/day topotecan hydrochloride via gavage, iii) 3 mg/kg/d topotecan hydrochloride via gavage; and iv) positive control (3 mg/kg/day Azactidine via intraperitoneal injection at weeks 1, 3 and 4, 5 days a week). Tumor development were assessed weekly using the IVIS® imaging platform (Xenogen Corp.). The weight of the mice was measured every three days. The mice were sacrificed by cervical dislocation when demonstrating signs of imminent death, such as reduced mobility or temperature. Data were analyzed using the Living Image 4.4 (PerkinElmer, Inc.) and Xenogen IVIS 100 (Xenogen Inc.).

***Hematoxylin and eosin (H&E) staining.*** Tissue samples were fixed in 10% neutrally buffered formaldehyde for one day at room temperature, dehydrated using an increasing ethanol series and then embedded in paraffin. The paraffin-embedded samples were sectioned at a thickness of 4 µm. The sections were then subjected to H&E staining at room temperature for 5 min per stain to visualize the nucleus and cytoplasm. The stained sections were assessed for any histopathological changes using a light microscope (Leica Microsystems) and imaged.

***Western blotting experiment of tumor tissue.*** Previously collected tumor tissues were cut and placed in tissue-crushing tubes, and 2-3 iron beads and RIPA buffer were added to each tube. The tissue was homogenized using a tissue crusher until a homogenous solution was obtained. The homogenate was centrifuged at 6,037.2 x g at 4°C for 20 min, and the resulting supernatant was transferred to microcentrifuge tubes. Protein content in the supernatant was determined using a BCA kit, and protein samples were prepared for western blotting analysis according to the aforementioned method.

***Immunohistochemistry (IHC).*** Tumor tissue samples from both the xenograft and *in situ* models were fixed in a 4% paraformaldehyde solution overnight at 4°C and subsequently sectioned at a thickness of 4 µm. The sections were then

subjected to overnight staining using primary antibodies against p-STAT5 (1:100; cat. no. ab32364; Abcam), STAT5 (1:100; cat. no. ab230670; Abcam), Cleaved Caspase 3 (1:100; cat. no. AF7022; Affinity Biosciences) and Ki-67 (1:100; cat. no. ab1667; Abcam) at 4°C. HRP labeled goat anti-rabbit/mouse secondary antibody (cat. no. PR30009; Proteintech Group, Inc.) incubated at 37°C for 30 min. Anti-biotin protein-biotin peroxidase complexes were then utilized according to the manufacturer's instructions, followed by colorimetric detection using DAB (3,3'-Diaminobenzidine). Finally, hematoxylin was used to counterstain the sections at room temperature for 2 min, which were then mounted with coverslips. Sections were imaged using an Olympus BX53 biological microscope and images were assessed using Image-Pro Plus 6.0 (media Cybernetics, Inc.).

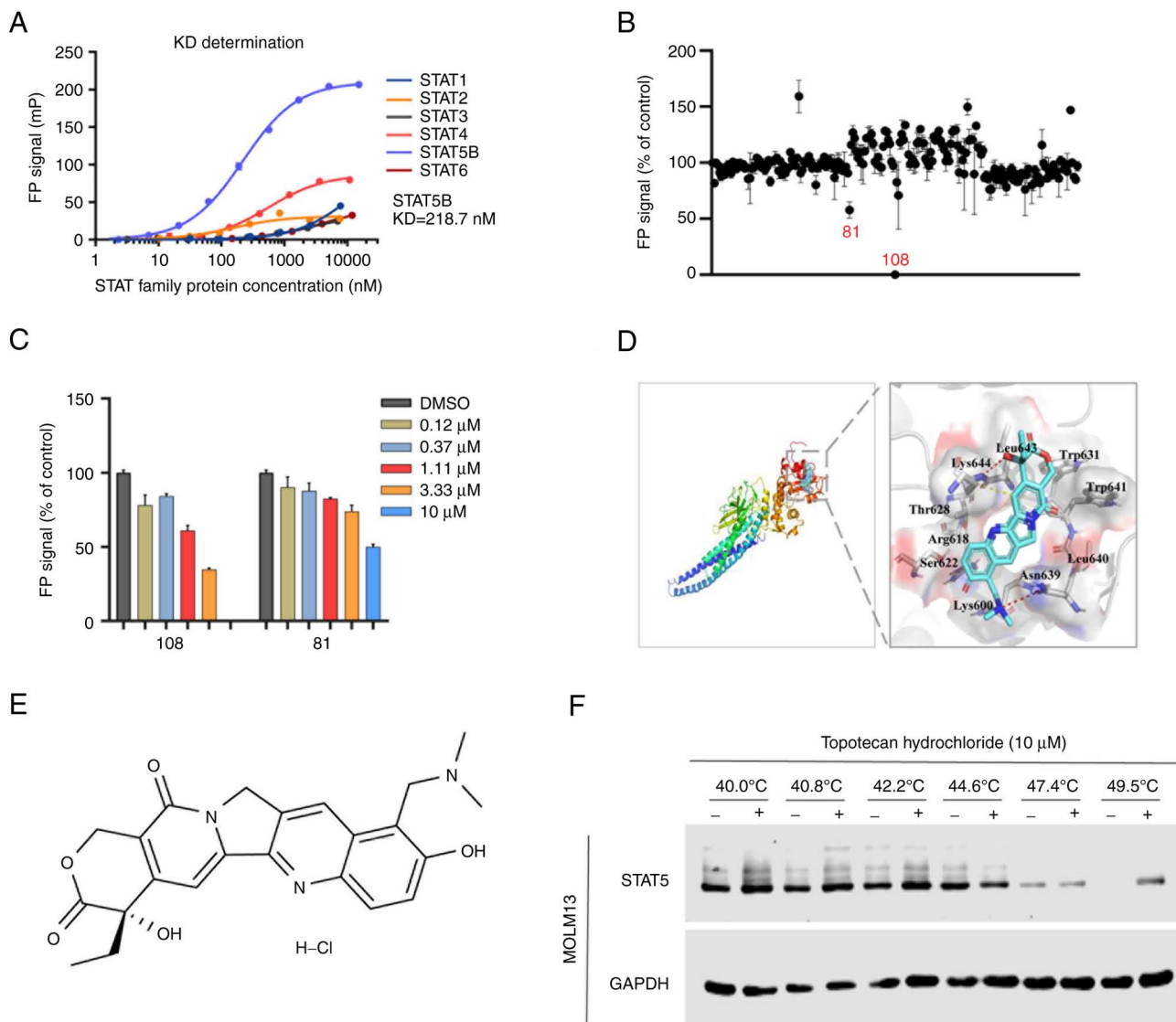
***Statistical analyses.*** Experiments were performed with ≥3 replicates and statistical analyses were performed using Student's t-test or one-way ANOVA followed by Dunnett's post-hoc test. The data are presented as mean ± SD. The evaluations were performed using Microsoft Excel 2019 (Microsoft Corporation) and GraphPad (version 7.0; Dotmatics). P<0.05 was considered to indicate a statistically significant difference.

## Results

***Topotecan hydrochloride binds to and inhibits STAT5.*** Previous studies have reported that STAT5b is the driving force behind maintenance and self-renewal of HSC and LSCs (8); therefore, STAT5b was assessed in the present study. To screen small molecule inhibitors targeting STAT5 function, a homogeneous method based on fluorescence polarization (FP) *in vitro* was developed. Due to the high homology of the STAT family, the STAT1, STAT2, STAT3, STAT4, STAT5b and STAT6 proteins were first purified and used to confirm system specificity. The dissociation constants (KD) of the STAT family (STAT1-6) were determined and it was demonstrated that the selective activity of the FP system against STAT5 was the best (STAT3 KD <50 nM; STAT4 KD <100 nM; STAT5 KD=218.7 nM) (Fig. 1A), so the system was considered to be specific to STAT5. After that, the stability of the system was tested, and the optimal incubation temperature, time and strength were determined. It was demonstrated that the influence of DMSO on the system was negligible (Fig. S1A). Furthermore, incubation at room temperature for 1 h was the best condition for the combination of protein and peptide (Fig. S1C), which confirmed the stability of the FP system (Fig. S1B).

Using the FP-STAT5 system, a compound library was screened and two potential inhibitors, namely compounds 81 and 108, with inhibitor constant values of 10 and 1-3 µM, respectively, were identified. Compound 108 (topotecan hydrochloride) demonstrated the most potent inhibitory activity (50% inhibition; 81, 10 µM; and 108, <10 µM) and was selected as the lead candidate for further experimental investigation (Fig. 1B and C).

To evaluate the binding pattern between topotecan hydrochloride and the STAT5-SH2 domain, an *in silico* molecular simulation docking experiment was performed. The molecular model of interaction between topotecan hydrochloride and



**Figure 1.** Topotecan hydrochloride specifically binds STAT5. (A) The system specificity of STAT5-FP was tested by binding with STAT family proteins. (B and C) Following incubation of purified STAT5 protein with its labeled STAT5 phosphopeptide [5-carboxyfluorescein GYACHTUNG(TRENNUNG)(PO3H2) LVLDKW] for 1 h at room temperature, FP signals of two candidate compounds at different drug concentrations were tested and analyzed to identify molecular inhibitory activity. (D) Interaction of topotecan hydrochloride with STAT5. Molecular docking models demonstrated that topotecan hydrochloride bound to multiple amino acids in the SH2 domain of STAT5. (E) The chemical structure of topotecan hydrochloride. (F) Cellular thermal shift analysis, MOLM13 cells were plated and treated with 10  $\mu$ M of topotecan hydrochloride for 1 h. The samples were put on the PCR instrument and heated at different temperatures for 3 min. After that, liquid nitrogen was used to freeze and thaw samples twice, and cell suspension samples were collected and STAT5 protein levels assessed. FP, fluorescence polarization; KD, dissociation constant.

STAT5-SH2 demonstrated that the hydroxyl part of the six-member lactone ring of topotecan hydrochloride and the dis-substituted amino part of the benzene ring were bonded to Asn639 and Lys644 of the STAT5-SH2 domain by hydrogen bonds (Fig. 1D and E). To confirm whether topotecan hydrochloride specifically targeted STAT5, CETSA was performed. After treating cells with 10  $\mu$ M topotecan hydrochloride for 1 h, western blotting analysis demonstrated that the thermal denaturation temperature of STAT5 in the control group treated with DMSO was 47.4°C. However, after treatment with topotecan hydrochloride, the thermal denaturation temperature increased to 49.5°C, with no apparent effect on the stability of GAPDH expression (Fig. 1F). The increase in thermal stability of the STAT5 target protein indicated that topotecan hydrochloride specifically bound to STAT5. This

indicated that topotecan hydrochloride was an effective small molecule inhibitor of STAT5.

**Topotecan hydrochloride suppresses STAT5 activation in AML.** To assess the potential of topotecan hydrochloride as a STAT5 inhibitor, its inhibitory effect on STAT5 activation and downstream signaling were assessed in AML cells, including the MOLM13 (FLT3-ITD<sup>+</sup>), NB4 and KG1 cell lines. Following treatment with a range of concentrations of topotecan hydrochloride for 24 h, western blotting demonstrated that topotecan hydrochloride effectively blocked STAT5 phosphorylation in the three cell lines, which indicated its potential as a STAT5 inhibitor (Fig. 2A). In addition, as the concentration of topotecan hydrochloride increased, the protein expression level of the downstream STAT5 gene, CMYC, markedly decreased,



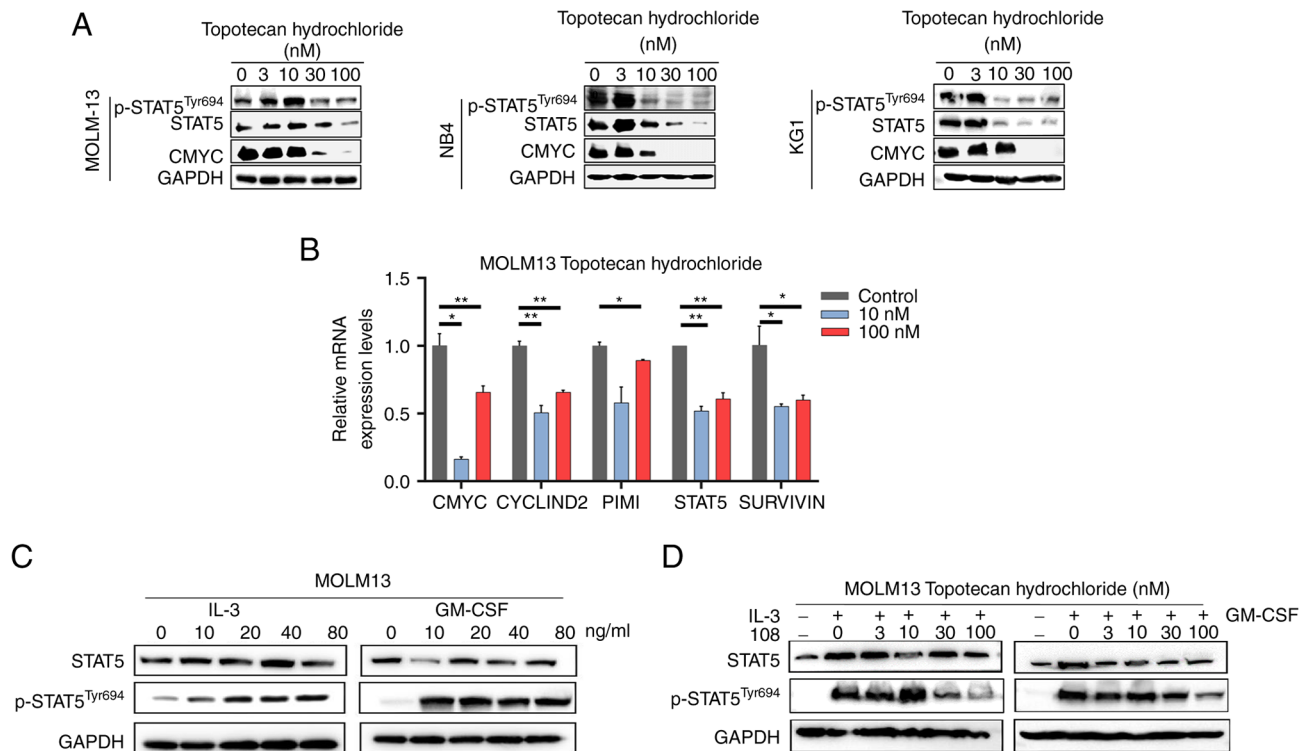


Figure 2. Topotecan hydrochloride inhibited STAT5 activation in acute myeloid leukemia cells. (A) MOLM13, NB4 and KG1 cells were treated a range of concentrations of topotecan hydrochloride for 24 h. Protein expression levels of p-STAT5, CMYC and STAT5 were assessed using western blotting with GAPDH as a control. (B) MOLM13 cells were treated with topotecan hydrochloride or DMSO (control) for 16 h, and the mRNA expression levels of STAT5 specific target genes were analyzed using reverse transcription-quantitative PCR. (C) Different concentrations of IL-3 and GM-CSF were added to stimulate the cells for 20 min. Then the cells were collected and western blotting was used to assess protein expression levels. (D) Cells were starved for 24 or 48 h in medium without fetal bovine serum and then treated with a range of concentrations of topotecan hydrochloride for 24 h. Cells were then stimulated with 5 ng/ml IL-3 and GM-CSF for 20 min and subjected to western blotting. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$  and \*\* $P < 0.01$ . GM-CSF, granulocyte-macrophage colony stimulating factor; ns, not significant; p, phosphorylated.

which indicated that it could block the downstream STAT5 signaling pathway. Moreover, RT-qPCR assays using MOLM13 cells demonstrated that topotecan hydrochloride significantly reduced the mRNA expression levels of STAT5-specific target genes, such as CMYC, CYCLIND2, PIMI and STAT5. Notably, a significant decrease in the mRNA expression levels of SURVIVIN suggested that topotecan hydrochloride could induce apoptosis (Fig. 2B). In summary, the results of the present study indicated that topotecan hydrochloride has the potential to inhibit STAT5 activation, block downstream signaling and induce apoptosis in AML cells.

IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF) are known to activate STAT5 via receptor-dependent pathways, inducing the expression of STAT5 target genes through JAK2 and STAT5 signaling pathways (40,41). The present study demonstrated that with the addition of IL-3 and GM-CSF cytokines, the phosphorylation of STAT5 in MOLM13 cells increased notably as the concentrations of IL-3 and GM-CSF increased (Fig. 2C). Cells were treated with topotecan hydrochloride for 24 h and stimulated with IL-3 and GM-CSF for 20 min, it was observed that topotecan hydrochloride effectively inhibited STAT5 phosphorylation even when IL-3 and GM-CSF activated STAT5 expression, which suggested that it could block STAT5 activation (Fig. 2D). The results demonstrated that topotecan hydrochloride could specifically reduce STAT5 phosphorylation and downstream gene expression, thereby inhibiting STAT5 activation in AML cells.

*Topotecan hydrochloride induces apoptosis and cell cycle arrest in AML cells.* MOLM13 (FLT-ITD<sup>+</sup>), KG1, HL-60 and NB4 cells demonstrated high protein expression levels of p-STAT5 (Fig. 3A), MOLM13 and KG1 (as the most commonly used in the literature (32,42) cells were selected for further study. The MTS cell viability assay demonstrated that topotecan hydrochloride had a strong inhibitory activity against AML cells, with  $IC_{50}$  values ranging from 11-21 nM (Fig. 3B). Topotecan hydrochloride demonstrated poor cell activity in control cell lines (HUVECs) and had no inhibitory effect on normal monocytes (PBMC), which indicated that topotecan hydrochloride specifically inhibited the proliferation of STAT5-expressing AML cells (Fig. 3B). The results demonstrated that topotecan hydrochloride effectively inhibited AML cell growth by targeting STAT5.

AML cells were treated with the existing STAT5 inhibitors AC-4-130 and Pimozide, and MTS cell viability was determined. The results demonstrated that the  $IC_{50}$  values for AC-4-130 were 6.58-15.21  $\mu$ M and those for Pimozide were 19.19-62.17  $\mu$ M. The two inhibitors had poor cellular activity and topotecan hydrochloride demonstrated a ~1,000-fold greater cellular activity compared with them (Fig. 3C and D).

In MOLM13 and KG1 cells, expression of Cleaved Caspase 3, the apoptosis marker, markedly increased in an apparently concentration-dependent manner, which suggested

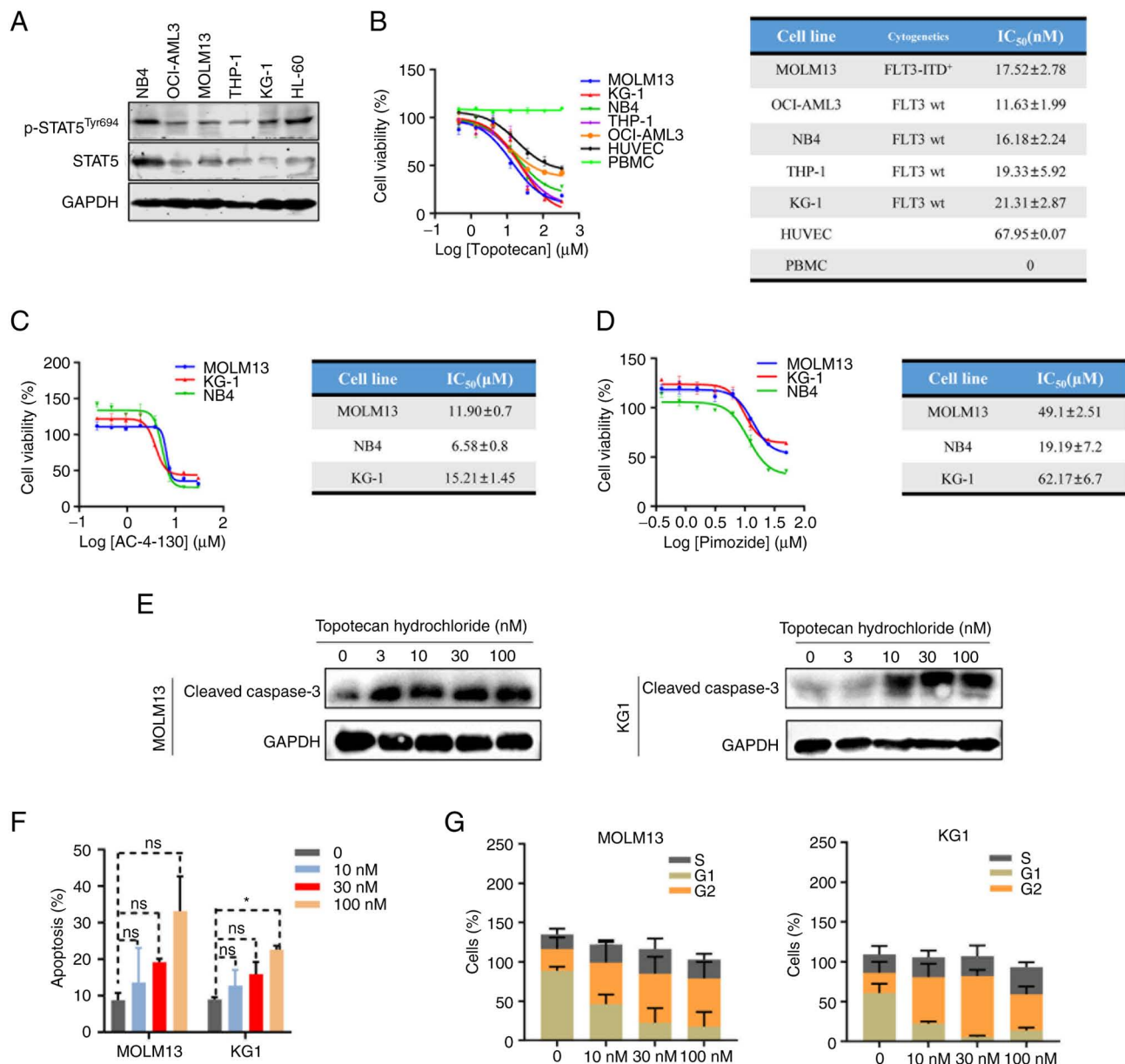


Figure 3. Topotecan hydrochloride inhibits AML by inducing apoptosis. (A) AML cell lines were treated with a range of concentrations of topotecan hydrochloride for 24 h and assessed using western blotting. (B) MTS viability assays were performed on AML and control cells treated for 72 h with topotecan hydrochloride or DMSO. IC<sub>50</sub> values were calculated using GraphPad. MTS viability assays were performed on AML cells treated for 72 h with (C) AC-4-130 or (D) Pimozide. IC<sub>50</sub> values were calculated using GraphPad. (E) MOLM13 and KG1 cells were treated with topotecan hydrochloride for 24 h, Cleaved Caspase 3 was detected using western blotting and GAPDH was used as a control. (F and G) After MOLM13 and KG1 cells were treated with a range of concentrations of topotecan hydrochloride or DMSO for 24 h, the cell cycle distribution was determined using propyl iodide staining. After 48 h of drug treatment, Annexin V/PI staining was used to detect apoptotic cells. Data are presented as mean ± standard deviation. \*P<0.05. ns, not significant; AML, acute myeloid leukemia.

that topotecan hydrochloride could induce apoptosis (Fig. 3E). The cell cycle and apoptosis of MOLM13 and KG1 cells treated with topotecan hydrochloride were analyzed, flow cytometry demonstrated that topotecan hydrochloride induced apoptosis and cell cycle arrest, with a marked decrease in the number of cells in G1 phase and a marked increase in G2/M phase (Figs. 3F and G, and S2A and B).

These results suggested that topotecan hydrochloride inhibited the proliferation of AML cells by inducing cell cycle arrest and apoptosis. In summary, topotecan hydrochloride targeted AML cells that expressed STAT5 and inhibited their proliferation through specific targeting of STAT5 pathways.

*Topotecan hydrochloride inhibit AML tumor development in vivo.* To assess the anti-tumor effect of topotecan hydrochloride *in vivo*, a subcutaneous tumor growth xenograft model using MOLM13 was established. The results demonstrated that after 30 days of treatment, the 1 mg/kg and 3 mg/kg topotecan hydrochloride groups demonstrated a significant reduction in tumor volume and inhibition of AML tumor growth, compared with the control (Fig. 4A and B). Upon killing the mice, the tumor was collected, imaged and weighed to record the tumor mass. A notable trend of reduced tumor volume and tumor mass was apparent. Throughout the experiment, the mice well-tolerated different doses of topotecan hydrochloride, and

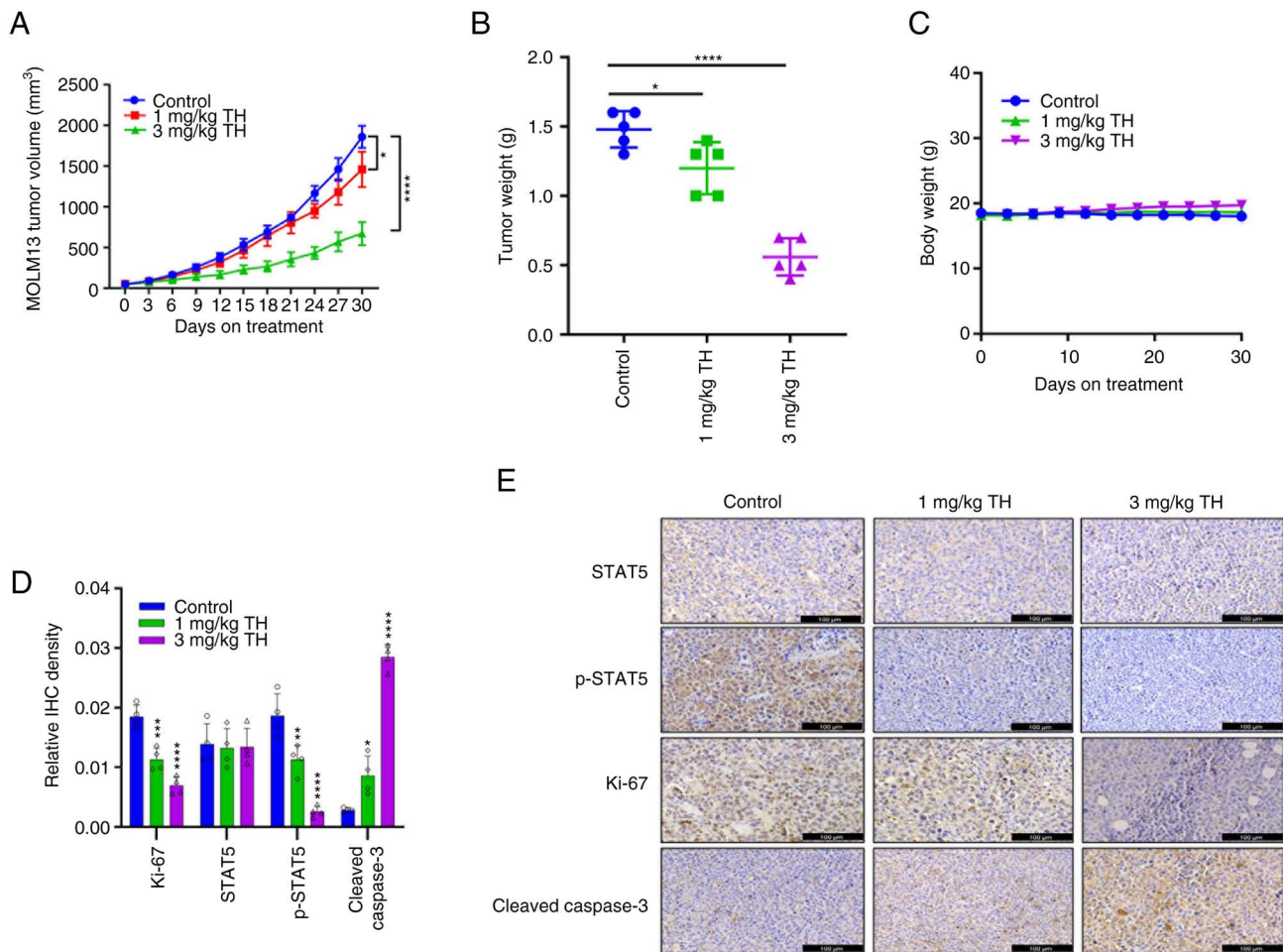


Figure 4. Topotecan hydrochloride inhibits acute myeloid leukemia tumor growth *in vivo*. MOLM13 cells were subcutaneously administered at a density of  $5 \times 10^6$  cells/mouse. After the tumor volume grew to 100–150 mm<sup>3</sup>, the animals were randomly grouped and treated according to group administration. Equal amounts of solvent (n=5), 1 mg/kg/day topotecan hydrochloride (n=5), 3 mg/kg/day topotecan hydrochloride (n=5), or 3 mg/kg/day Azacitidine (n=5) were injected intraperitoneally once a day for 5 days a week at weeks 1, 3 and 4. (A) The tumor volume was calculated as (length x width<sup>2</sup>)/2. (B) After the experiments, all the tumors were weighed. (C) Body weight was measured every three days. IHC analysis was performed on the different treatment groups for p-STAT5, STAT5, Ki-67 and Cleaved Caspase 3. The results were quantified (D) and imaged (E). Scale bar=100  $\mu$ m. Data are presented as mean  $\pm$  standard deviation. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 and \*\*\*\*P<0.0001. TH, topotecan hydrochloride; IHC, immunohistochemistry; p, phosphorylated.

body weight was maintained (Fig. 4C). In comparison with the control group, H&E staining of the heart, liver, spleen, lungs and kidneys indicated no notable differences in the topotecan hydrochloride treatment group (Fig. S3). IHC results demonstrated that topotecan hydrochloride significantly inhibited STAT5<sup>Tyr694</sup> phosphorylation (Fig. 4D and E). Increasing drug concentration was observed to markedly increase Cleaved Caspase 3 levels, and topotecan hydrochloride significantly inhibited Ki-67 expression in tumor xenograft models, which indicated that topotecan hydrochloride induced apoptosis of AML cells *in vivo*. Overall, these results suggested that topotecan hydrochloride could significantly inhibit AML tumor growth, induce tumor cell apoptosis and inhibit STAT5 phosphorylation *in vivo*.

**Topotecan hydrochloride inhibits leukemic metastasis in vivo.** Topotecan hydrochloride was demonstrated to be effective in an AML subcutaneous tumor growth xenograft model; therefore, a MOLM13/Luc metastasis model was used to assess the effectiveness of topotecan hydrochloride in inhibiting leukemia metastasis and tumor growth *in vivo*. Azacitidine is

a positive control agent and is a first-line chemotherapy agent in elderly patients with AML who are not suitable for intensive therapy (43), which was approved in the European Union in 2017 for the treatment of adult AML.

MOLM13 cells expressing luciferase were injected into NOD/SCID mice through the caudal vein. Tumor formation *in vivo* was observed by two rounds of bioluminescence imaging 4–5 days after inoculation, tumor metastasis was monitored once a week after inoculation. The topotecan hydrochloride treatment group (1 and 3 mg/kg) markedly inhibit leukemia metastasis (Fig. 5A) and markedly reduced tumor growth and tumor volume, and the anti-tumor effect of 3 mg/kg topotecan hydrochloride was notably greater than that of Azacitidine. The body weight of mice in both the administration and control groups demonstrated a slight, steady increase (Fig. 5B). In comparison with the control group, H&E staining of the spleen demonstrated no significant difference in the effects of topotecan hydrochloride on the organs, which indicated that topotecan hydrochloride had low toxicity and good safety in mice at a dose of 1–3 mg/kg (Fig. S4). IHC demonstrated markedly decreased protein expression levels of



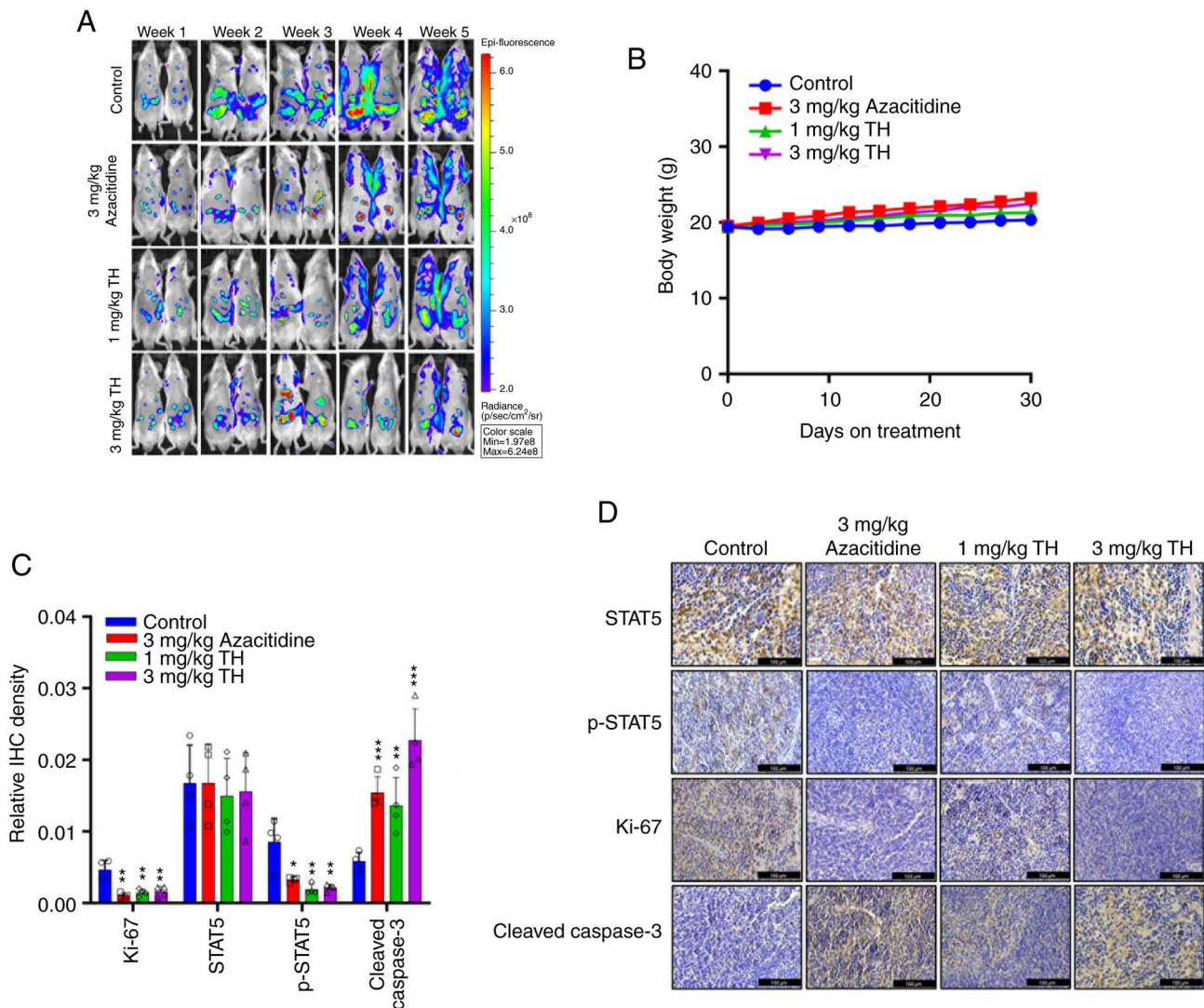


Figure 5. Topotecan hydrochloride inhibits tumor growth *in situ* in an acute myeloid leukemia model. MOLM13/Luc cells ( $1 \times 10^6$ ) were injected into the tail vein of pre-irradiated (2.5 Gy) NOD/SCID mice ( $n=5$  per group). The mice were then monitored for seven days and then treated with topotecan hydrochloride or Azacitidine for five weeks. (A) Tumor growth was assessed by weekly bioluminescence imaging. (B) Body weights of mice were measured ( $n=5$ ). IHC analysis was performed on the different treatment groups for p-STAT5, STAT5, Ki-67, and Cleaved Caspase 3. The results were (C) quantified and (D) imaged. Scale bar=100  $\mu$ m. Data are presented as mean  $\pm$  standard deviation. \* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$  ns, not significant; TH, topotecan hydrochloride; IHC, immunohistochemistry; p, phosphorylated.

STAT5, significantly decreased p-STAT5 and Ki-67 expression levels, and significantly increased Cleaved Caspase 3 in the 3 mg/kg topotecan hydrochloride group compared with the control (Fig. 5C and D), which suggested that topotecan hydrochloride inhibited phosphorylation and promoted apoptosis of AML cells *in vivo*.

## Discussion

The death rate from AML increased every year between 2017-2022, and the recurrence of AML is one of the reasons for treatment failure. There is an urgent need to develop new therapeutic strategies to improve the prognosis of AML patients (3). In recent years, the development of small-molecule targeted drugs has become a promising strategy for AML treatment, particularly for older patients (>68 years) who cannot tolerate high-intensity chemotherapy (6). This approach offers new treatment options for this population. Although inhibition

of the JAK/STAT signaling pathway is a promising strategy for inhibiting tumor growth, targeting this protein can be challenging (44).

In the present study, topotecan hydrochloride, a new STAT5 inhibitor, was identified using FP system screening, and through computer docking and CETSA experiments, it was demonstrated that topotecan hydrochloride directly combined with STAT5. Topotecan hydrochloride has good activity in cells at the nanomolar level. Topotecan hydrochloride selectively inhibits the activation and phosphorylation of STAT5 in AML cells and blocks the formation of dimers, which inhibits the growth and proliferation of AML. In addition, it was demonstrated that topotecan hydrochloride showed good anti-tumor activity in mice xenograft model via inhibition of STAT5 signaling.

It has previously been reported that the action of STAT5 small molecule inhibitors against AML proliferation is not good, at the micromolar level (45-47), however, topotecan

hydrochloride demonstrated good activity in AML cells, with a 1,000-fold increase in cell activity compared with other STAT5 inhibitors (AC-4-130 and Pimozide). Furthermore, topotecan hydrochloride was demonstrated to impede the phosphorylation of STAT5 and hinder dimer formation in AML cells, including FLT3-ITD<sup>+</sup> AML cells, which suggested its potential as an effective inhibitor of AML resistance and recurrence. However, there are still certain questions that need further study. Firstly, the specific binding sites of topotecan hydrochloride to STAT5 need to be elucidated to gain a better understanding of the molecular mechanisms involved. Proteins with different domain fragments should be purified and used to confirm the specific binding position of topotecan hydrochloride and STAT5 through methods such as microscale thermophoresis and surface plasmon resonance. Secondly, given that FLT3-ITD<sup>+</sup> AML cells are more sensitive to STAT5 inhibition, the specific inhibitory mechanism of STAT5 on FLT3-ITD<sup>+</sup> AML cells should be studied in the later stages of cell growth. Thirdly, the mechanism of drug resistance in AML should be further assessed.

The design and development of STAT5 inhibitors could lay a foundation for further development of FLT3-ITD<sup>+</sup> AML compounds with clinical value. STAT5 inhibitors not only represent a new therapeutic approach, but also indicate the potential undefined functions of STAT5 in AML cells. Overall, the present study demonstrated an advance in the development of treatments for AML and highlighted the potential of targeted therapies to combat drug resistance and improve patient outcomes.

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## Availability of data and materials

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

## Authors' contributions

JL, BT, ZS and YM designed the work, acquired data and interpreted the results. GL performed the statistical analysis. JL and ZS drafted the manuscript. JL, BT and YM confirm the authenticity of all the raw data.

## Ethics approval and consent to participate

All *in vivo* experiments were approved by the Animal Ethics Committee of Shanghai Fengxian District Central Hospital (approval no. 6600).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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