

Stattic suppresses p-STAT3 and induces cell death in T-cell acute lymphoblastic leukemia

CHIA-LING LI^{1,2}, HAN-YU CHEN², JIIN-CHERNG YEN³, SHENG-JIE YU^{4,6}, TING-YU CHOU⁴,
SIH-WEN YEH¹, HUAI-YU CHUANG⁷ and FANG-LIANG HUANG^{1,2,8,9}

¹Children's Medical Center, Taichung Veterans General Hospital, Taichung 407, Taiwan, R.O.C.; ²Department of Physical Therapy, Hung-Kuang University, Taichung 433, Taiwan, R.O.C.; ³Institute of Pharmacology, College of Medicine, National Yang Ming Chiao Tung University, Taipei 112, Taiwan, R.O.C.; ⁴Department of Medical Research, Taichung Veterans General Hospital, Taichung 407, Taiwan, R.O.C.; ⁵Institute of Biomedical Sciences, College of Life Sciences, National Chung Hsing University, Taichung 402, Taiwan, R.O.C.; ⁶Integrated Care Center of Psoriatic Disease, Taichung Veterans General Hospital, Taichung 407, Taiwan, R.O.C.; ⁷Department of Pathology, Tri-Service General Hospital Penghu Branch, Magong, Penghu County 880, Taiwan, R.O.C.; ⁸Department of Post-Baccalaureate Medicine, College of Medicine, National Chung Hsing University, Taichung 402, Taiwan, R.O.C.; ⁹Institute of Biomedical Sciences, MacKay Medical College, New Taipei 252, Taiwan, R.O.C.

Received August 7, 2024; Accepted November 15, 2024

DOI: 10.3892/mmr.2024.13416

Abstract. The present study investigated the therapeutic potential of Stattic, a selective inhibitor of STAT3, in treating T-cell acute lymphoblastic leukemia (T-ALL). The effects of Stattic on cell viability, STAT3 phosphorylation, apoptosis and autophagy in T-ALL cell lines, and on tumor growth in a xenograft mouse model of T-ALL, were assessed. Methods, including the Cell Counting Kit-8 assay for cell viability, propidium iodide/Annexin V staining for apoptosis detection, western blotting for protein expression analysis, and a xenograft mouse model for evaluating *in vivo* tumor growth, were employed. The results showed that Stattic effectively reduced cell viability in a dose-dependent manner, with significant reductions observed at concentrations of 1.25 μ M and above in CCRF-CEM cells (IC_{50} =3.188 μ M) and at 2.5 μ M and above in Jurkat cells (IC_{50} =4.89 μ M) after 24 h of treatment. Concurrently, Stattic significantly suppressed the expression of phosphorylated STAT3, indicating its mechanism of action as a STAT3 pathway inhibitor. Furthermore, Stattic treatment induced both apoptosis and autophagy in CCRF-CEM and Jurkat cells, as evidenced by the respective upregulation of

cleaved caspase-3 and LC3B. In a xenograft mouse model of T-ALL, Stattic markedly inhibited tumor growth, with the greatest effect occurring at the highest dose of 30 mg/kg. These results suggested that Stattic holds promise as a therapeutic agent in T-ALL by modulating key pathways involved in cell survival and proliferation. In conclusion, Stattic exhibited a significant therapeutic potential for T-ALL via a dose-dependent reduction of cell viability, inhibiting STAT3 phosphorylation, and promoting both apoptotic and autophagic cell death; however, further studies are required before clinical application.

Introduction

Acute lymphoblastic leukemia (ALL) is a hematological malignancy characterized by an abundance of immature lymphocytes, composed of 80-85% B cells and 20-25% T cells. This condition leads to the arrest of differentiation and abnormal proliferation of lymphocytes (1). ALL is the most prevalent form of cancer in children, accounting for ~30% of all childhood malignancies. Among acute leukemias, the incidence of ALL is five-times higher than that of acute myeloid leukemia (2). ALL is a significant global health concern, with an average incidence rate ranging from 0.4 to 2 per 100,000 individuals. The disease primarily affects children <15 years old, with the highest rates observed in high-income countries due to advanced diagnostic capabilities. Male individuals are more frequently affected than female individuals, with an incidence rate of 2.66 per 100,000 compared with 1.92 for female individuals. While high-income regions have successfully reduced mortality rates through improved treatment protocols, low-income countries continue to face higher death rates due to limited healthcare resources and late diagnoses. Addressing this disparity remains critical in the global fight against ALL (3). Notably, ALL is the most common type of cancer that occurs in childhood, with a peak occurrence

Correspondence to: Mr. Huai-Yu Chuang, Department of Pathology, Tri-Service General Hospital Penghu Branch, 90 Qianliao, Magong, Penghu County 880, Taiwan, R.O.C.
E-mail: airadam1983@gmail.com

Dr Fang-Liang Huang, Children's Medical Center, Taichung Veterans General Hospital, 1650 Taiwan Boulevard Sect. 4, Taichung 407, Taiwan, R.O.C.
E-mail: flhuang.ped@gmail.com

Key words: apoptosis, autophagy, Stattic, STAT3, T-cell acute lymphoblastic leukemia

between the ages of 2 and 5 years, as well as after the age of 50 years, with ~60% of cases occurring in individuals <20 years old (4). However, there is no significant sex difference in ALL incidence (5,6). Although its 5-year survival rate is ~90%, 20% of children with ALL experience relapse with a poor prognosis (7); in adults, the relapse rate is higher, reaching 40-50% (8,9). Therefore, continuous efforts are required to improve ALL treatment. Furthermore, children with ALL exhibit poorer social, physical and emotional health compared with their age-matched peers and siblings (10); these children may experience depression, anxiety and attention problems (11,12). Current ALL treatments primarily involve combination chemotherapy using high-dose methotrexate (MTX), mercaptopurine (6-MP) and other drugs, followed by the regular oral or injectable administration of anticancer drugs, such as dexamethasone (DEX), vincristine, cytarabine, Endoxan, 6-MP and MTX (13-16). Recurrence can occur due to the presence of residual cancer cells that are difficult to detect in the body after treatment. When these residual cancer cells proliferate, the disease re-emerges; thus, there remains an unmet medical need for treatment. In addition, unresolved medical issues, which include the high risk of relapse after the first remission and refractory disease after relapse (17), necessitate further investigation.

Previous studies have detected the sustained activation of the JAK2/STAT3 signaling pathway in various types of human cancer, including blood cancer, and its association with poor prognosis (18,19). The small molecule compound Stattic is a potent STAT3 inhibitor, which selectively blocks the SH2 domain, regardless of its phosphorylation status (20,21). This signal transduction is selectively inhibited by Stattic, together with the activation, dimerization and nuclear translocation of STAT3. The consequence of this inhibition is a greater apoptotic rate of STAT3-dependent cancer cells (22). Based on previous studies, the present study aimed to determine whether Stattic can achieve anticancer effects by regulating the role of STAT3 in ALL. The aim was to reveal the cellular and molecular mechanisms underlying the anticancer effects of Stattic. Understanding the related role and mechanisms of Stattic in ALL may have extensive clinical and immunological significance, and the results of the present study may aid in the development of effective treatments for patients with ALL and provide novel insights into the basic treatment of ALL.

Materials and methods

Cell culture. The human T-cell ALL (T-ALL) cell lines CCRF-CEM and Jurkat (American Type Culture Collection) were maintained in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (HyClone; Cytiva), 1 mM sodium pyruvate (HyClone; Cytiva), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO₂ at 37°C.

Reagents. Stattic (cat. no. HY-13818; MedChemExpress), a selective inhibitor of STAT3, and dimethyl sulfoxide (DMSO), which was used as a vehicle control, were procured from Sigma-Aldrich; Merck KGaA. For cell treatments, Stattic was dissolved in DMSO for stock preparation. In the *in vitro*

experiments, the vehicle control group received a final DMSO concentration of 0.05%, while the 5, 2.5 and 1.25 µM Stattic groups received DMSO concentrations of 0.05, 0.025 and 0.0125%, respectively.

Cell viability assay. The cytotoxic effects of Stattic on CCRF-CEM and Jurkat cells were assessed using the Cell Counting Kit-8 (CCK-8; cat. no. 96992; Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. Briefly, a total of 1x10⁵ cells/well were seeded in 200 µl culture medium in a 96-well cell culture plate, and were then treated at 37°C for 24 h with various concentrations of Stattic (0.625, 1.25, 2.5, 5 and 10 µM) or an equivalent volume of the vehicle control. Subsequently, CCK-8 solution was added, and plates were incubated for another 2-3 h. Absorbance at 450 nm was then measured using a microplate reader (Enspire 2300-0000; PerkinElmer, Inc.).

Western blot analysis. For western blot analysis, cells were treated prior to collection. In the dose-dependent experiment, cells were treated with DMSO or different concentrations of Stattic (1.25, 2.5 and 5 µM) for 24 h. In the time-course experiment, cells were treated with DMSO or 5 µM Stattic for 8, 16 and 24 h. After treatment, the cells were first washed with PBS prior to collection, and were then lysed in ice-cold Tris buffer (50 mM, pH 7.5) containing the following: 5 mM EDTA, 300 mM NaCl, 0.1% Igepal, 0.5 mM NaF, 0.5 mM Na₃VO₄, 0.5 mM PMSF and antiprotease mixture (Roche Molecular Diagnostics), and centrifuged at 13,000 x g for 10 min at 4°C. Protein concentrations were determined according to the Bradford procedure. Equal amounts of protein (25 µg) were then separated by SDS-PAGE on 10 and 12% gels, and were transferred to PVDF membranes. After blocking with 5% non-fat milk dissolved in PBS at room temperature for 1 h, the membranes were incubated with primary antibodies against p-STAT3 (1:1,000; cat. no. 9145S; Cell Signaling Technology, Inc.), STAT3 (1:1,000; cat. no. 30835S; Cell Signaling Technology, Inc.), pro-caspase-3 (1:1,000; cat. no. 9662S; Cell Signaling Technology, Inc.), cleaved caspase-3 (1:1,000; cat. no. 9662S; Cell Signaling Technology, Inc.), LC3B (1:1,000; cat. no. 83506S; Cell Signaling Technology, Inc.), p62 (1:1,000; cat. no. 5114; Cell Signaling Technology, Inc.), Bcl-2 (1:1,000; cat. no. 26593-1-AP; Proteintech Group, Inc.), PARP-1 (1:1,000; cat. no. sc-74470; Santa Cruz Biotechnology, Inc.), ATG5 (1:1,000; cat. no. sc-133158; Santa Cruz Biotechnology, Inc.), BECN1 (1:500; cat. no. sc-11427; Santa Cruz Biotechnology, Inc.) and β-actin (1:5,000; cat. no. 3700S; Cell Signaling Technology, Inc.), followed by incubation with an antimouse IgG, HRP-linked secondary antibody (1:7,000; cat. no. 7076P2; Cell Signaling Technology, Inc.) or an anti-rabbit IgG, HRP-linked secondary antibody (1:7,000; cat. no. 7074P2; Cell Signaling Technology, Inc.). The ATG5 antibody used in the present study can detect the ATG5-ATG12 conjugate with a molecular weight of ~50 kDa. Bands were visualized using an Enhanced Chemiluminescence Detection Kit (MilliporeSigma) and the Alliance Q9 (Uvitec Ltd.). The protein expression levels were normalized to β-actin. The band intensity was measured using ImageJ software (version 1.50i; National Institutes of Health).

Flow cytometric analysis. Apoptosis was quantified based on Annexin V-FITC and propidium iodide (PI) double staining. Briefly, 4×10^5 CCRF-CEM cells and 3×10^5 Jurkat cells were treated with $5 \mu\text{M}$ Stattic or DMSO in a 24-well plate for 24 h. After treatment, the cells were collected and stained using the Annexin V-FITC/PI apoptosis kit according to the manufacturer's protocol (Elabscience Bionovation Inc.). Stained cells were analyzed by flow cytometry within 1 h of staining. Fluorescence intensities were measured using a flow cytometer (FACSCanto II; BD Biosciences) with BD FACSDiva software (version 8.0.1; BD Biosciences). Each experiment was performed in duplicate, at least three times independently.

Xenograft T-ALL mouse model. Female NOD/SCID mice (age, 9 weeks; weight, 18–22 g) were procured from the National Laboratory Animal Center (Taipei, Taiwan). The mice were maintained in an individually ventilated caging system, adhering to a 12-h light/dark cycle, at a temperature of $22 \pm 2^\circ\text{C}$ and a humidity of 50–70%, with *ad libitum* access to food and water. All experimental protocols were approved by the Animal Care and Use Committee of the Taichung Veterans General Hospital (IACUC no. La-1132052; Taichung, Taiwan). To create tumors, 1×10^6 CCRF-CEM cells suspended in a 1:1 mixture of BD Matrigel™ (cat. no. 356231; Corning, Inc.) and RPMI-1640 medium were subcutaneously injected into the flanks of each mouse. On day 1, T-ALL cells were subcutaneously injected into mice under gas anesthesia using isoflurane (induction, 4–5% isoflurane for 3 min; maintenance, 1–3% isoflurane for an additional 1 min). After the procedure, the mice were placed in a warm, dry environment and were continuously monitored for vital signs during recovery. The mice were returned to their original housing only after fully recovering from anesthesia. Post-surgery, the mice were examined at least once daily, monitoring the injection site for wound condition, including any signs of secretion, as well as assessing body weight, eating, urination and defecation. Analgesics were not used, as they could impact the experimental results; instead, physical pain management strategies, such as environmental enrichment items (e.g., toys), were provided. Mice received wooden sticks, paper houses and similar enrichment items. Mice were subsequently randomized into the following five groups ($n=6$ mice/group): Vehicle control group and four treatment groups, each receiving three different doses of Stattic (7.5, 15 and 30 mg/kg) or DEX (1 mg/kg ; Taiwan Biotech Co., Ltd.). Starting from day 6 after tumor cell injection, Stattic and DEX were administered by intraperitoneal injection three times a week; the volume of intraperitoneal injection per mouse was $300 \mu\text{l}$. Stattic was dissolved in DMSO for stock preparation. The final concentrations of DMSO within the administered Stattic doses (7.5, 15, and 30 mg/kg) were 0.75, 1.5 and 3%, respectively. For the vehicle control group, the injection consisted of normal saline containing 3% DMSO. Tumor sizes were measured using a digital caliper thrice weekly until the day of sacrifice and tumor volume was calculated using the following formula: Tumor volume = length $\times$ width². All mice were euthanized on day 23 for subsequent analyses. Mice were placed into a euthanasia chamber, which was gradually filled with CO_2 at a flow rate of 30% volume/min. After the gas infusion, the mice were observed for 3 min to ensure proper

euthanasia; the signs confirming death included the cessation of heartbeat, lack of respiratory activity and pupil dilation.

Statistical analysis. Data are presented as the mean $\pm$ SEM of at least three independent experiments. To compare differences between the treatment and control groups, statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Data were analyzed using GraphPad Prism software (version 6; Dotmatics). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Stattic inhibits the viability of CCRF-CEM and Jurkat cells in a dose-dependent manner. To assess the cytotoxic effects of Stattic on T-ALL cells, CCRF-CEM (Fig. 1A) and Jurkat cells (Fig. 1B) were treated with increasing concentrations of Stattic (0.625, 1.25, 2.5, 5 and $10 \mu\text{M}$) or vehicle control (DMSO) for 24 h. Cell viability was measured using the CCK-8 assay. In CCRF-CEM cells, Stattic treatment resulted in a significant, dose-dependent reduction in cell viability. A statistically significant reduction was observed at $1.25 \mu\text{M}$, and cell viability was further decreased at $2.5 \mu\text{M}$ and higher concentrations. The half maximal inhibitory concentration (IC_{50}) value for CCRF-CEM cells was determined to be $3.188 \mu\text{M}$, indicating that these cells were sensitive to Stattic-induced inhibition of viability (Fig. 1A). In Jurkat cells, a similar dose-dependent reduction in viability was observed; however, the inhibitory effect was less pronounced compared with in CCRF-CEM cells. Significant reductions in viability were detected at 5 and $10 \mu\text{M}$ concentrations. The IC_{50} value for Jurkat cells was $4.89 \mu\text{M}$, suggesting that Jurkat cells are slightly more resistant to Stattic than CCRF-CEM cells (Fig. 1B). These results indicated that Stattic may effectively reduce the viability of CCRF-CEM and Jurkat cells in a dose-dependent manner, with CCRF-CEM cells showing greater sensitivity. The observed differential sensitivity between the two cell lines highlights the potential for Stattic as a targeted therapeutic agent in T-ALL.

Stattic suppresses p-STAT3 levels in CCRF-CEM and Jurkat cells. To investigate the effect of Stattic on STAT3 signaling, the expression levels of p-STAT3 were examined in CCRF-CEM (Fig. 2A) and Jurkat cells (Fig. 2B) at 8, 16 and 24 h following treatment with $5 \mu\text{M}$ Stattic or DMSO. Total STAT3 and β -actin were used as loading controls. In CCRF-CEM cells, Stattic treatment resulted in a reduction in p-STAT3 levels over time. Notably, a significant decrease in p-STAT3 was observed at 24 h, indicating that Stattic effectively suppressed STAT3 phosphorylation with prolonged exposure. However, total STAT3 levels remained stable across all time points, suggesting that Stattic may specifically inhibit STAT3 activation without affecting its overall expression (Fig. 2A). In Jurkat cells, although p-STAT3 levels fluctuated, no statistically significant differences were observed at any of the time points compared with the DMSO-treated controls. Total STAT3 expression remained constant, similar to in CCRF-CEM cells (Fig. 2B). These findings indicated that Stattic may inhibit STAT3 activation more effectively in CCRF-CEM cells than in Jurkat cells, reflecting a differential

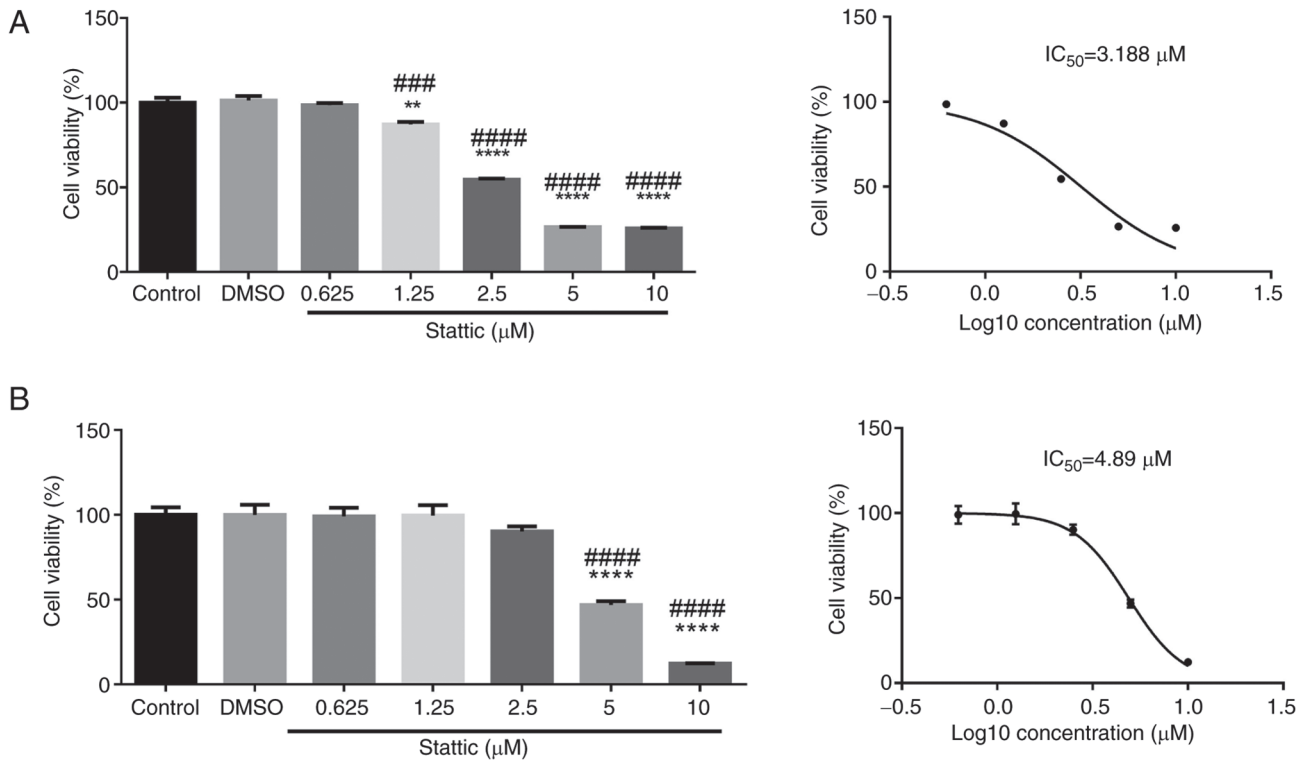


Figure 1. Static significantly inhibits T-cell acute lymphoblastic leukemia cell viability in a dose-dependent manner. The viability of (A) CCRF-CEM and (B) Jurkat cells was detected following treatment with different concentrations of Static (0.625–10 μM) for 24 h. Dose-response curves are shown. The IC_{50} values of Static are 3.188 μM in CCRF-CEM cells and 4.89 μM in Jurkat cells. The results are presented as the mean $\pm$ SEM ($n=3$). ** $P<0.01$, **** $P<0.0001$ vs. control group (untreated); *** $P<0.001$, **** $P<0.0001$ vs. vehicle control group (0.1% DMSO). IC_{50} , half maximal inhibitory concentration.

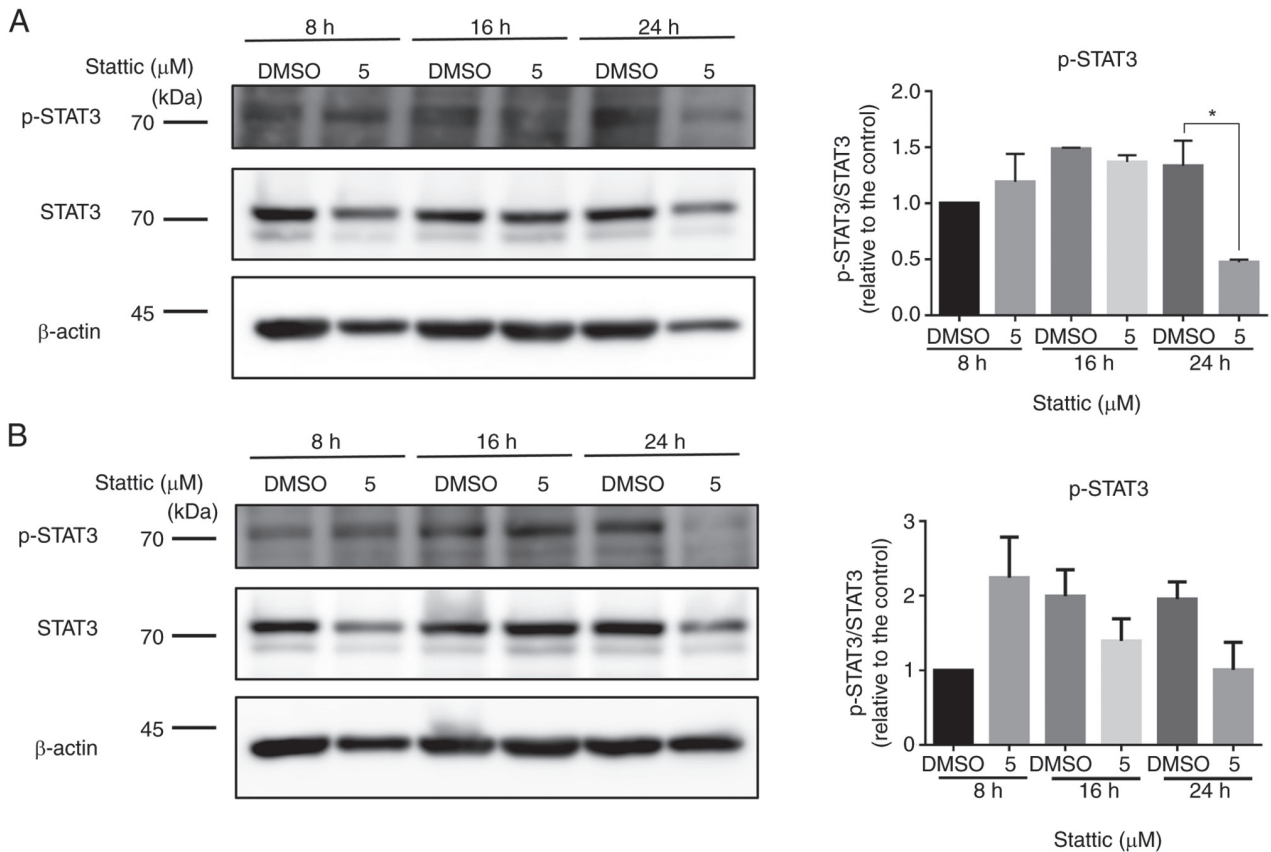


Figure 2. Static significantly inhibits p-STAT3 expression in T-cell acute lymphoblastic leukemia cells after 24 h of treatment. Western blot analysis of p-STAT3 and total STAT3 levels in (A) CCRF-CEM and (B) Jurkat cells treated with 5 μM Static or DMSO for 8, 16 and 24 h. β -actin was used as a loading control. Vehicle control group was treated with 0.05% DMSO. The results are presented as the mean $\pm$ SEM ($n=3$). * $P<0.05$. p-, phosphorylated.

response between the two T-ALL cell lines. The suppression of STAT3 phosphorylation suggested that Stattic may exert its anti-proliferative effects, at least in part, through the inhibition of STAT3-mediated signaling in CCRF-CEM cells.

Stattic induces apoptosis and autophagy-related changes in T-ALL cells. Western blot analysis was conducted to investigate the time-dependent effects of Stattic (5 μ M) on apoptosis and autophagy markers in two T-ALL cell lines: CCRF-CEM (Fig. 3A) and Jurkat (Fig. 3B). Both cell types were treated for 8, 16 and 24 h with Stattic or with DMSO as a control. In CCRF-CEM cells, an inhibition of pro-caspase-3 expression was observed at 8 and 24 h after Stattic treatment. Furthermore, a significant increase was detected in cleaved caspase-3 expression 16 h after Stattic treatment, indicating the activated apoptotic cascade. Stattic also induced a time-dependent upregulation of LC3B expression at 8, 16 and 24 h, suggesting enhanced autophagic activity. Meanwhile, p62 protein levels showed a decreasing trend at 24 h, although this change was not statistically significant, further supporting autophagy activation. Bcl-2 expression remained relatively stable, and full-length PARP-1 displayed a significant reduction at 8 and 16 h, reflecting apoptotic progression. Markers related to autophagy initiation, such as ATG5-ATG12 conjugate and BECN1, did not exhibit substantial changes during the observation period (Fig. 3A). In Jurkat cells, the apoptotic response to Stattic was less pronounced. While pro-caspase-3 levels remained relatively stable, cleaved caspase-3 exhibited a slight increase at 16 h. LC3B levels also demonstrated a significant increase at 8, 16 and 24 h. Similarly, p62 levels showed a decreasing trend at 24 h, indicating autophagy activation, though less prominent compared with in CCRF-CEM cells. Bcl-2 expression was significantly reduced at 8 h but remained unchanged thereafter. Full-length PARP-1 and autophagy-related proteins, including ATG5-ATG12 conjugate and BECN1, showed no significant changes across all time points (Fig. 3B). Together, these results indicated that Stattic could induce both apoptotic and autophagic processes in CCRF-CEM and Jurkat cells, with more robust effects observed in CCRF-CEM cells. The differential responses between the two cell lines highlight the potential variability in the sensitivity of T-ALL subtypes to Stattic treatment.

Stattic dose-dependently inhibits p-STAT3 expression in CCRF-CEM and Jurkat cells. To further explore the impact of Stattic on STAT3 signaling, CCRF-CEM (Fig. 4A) and Jurkat cells (Fig. 4B) were treated with increasing concentrations of Stattic (1.25, 2.5 and 5 μ M) or vehicle control (DMSO) for 24 h. The expression levels of p-STAT3 and total STAT3 were measured by western blotting, with β -actin serving as the loading control. In CCRF-CEM cells, Stattic reduced p-STAT3 levels in a dose-dependent manner. A marked reduction in p-STAT3 was observed at the 5 μ M concentration, with statistical significance indicated. However, total STAT3 protein expression remained unchanged across all treatment groups, suggesting that Stattic specifically inhibited STAT3 phosphorylation without affecting total STAT3 levels (Fig. 4A). Similarly, in Jurkat cells, p-STAT3 levels were reduced with increasing Stattic concentrations. A significant decrease was evident in response to the 5 μ M concentration,

while total STAT3 levels showed no major changes, indicating selective inhibition of phosphorylation by Stattic (Fig. 4B). These results indicated that Stattic may effectively inhibit STAT3 activation in a dose-dependent manner in both CCRF-CEM and Jurkat cells. The suppression of p-STAT3 without affecting total STAT3 levels further supports the role of Stattic as a selective inhibitor of STAT3 signaling, which could underlie its therapeutic potential in T-ALL.

Stattic modulates apoptosis and autophagy markers in CCRF-CEM and Jurkat cells in a dose-dependent manner. To investigate the effects of Stattic on apoptosis and autophagy, CCRF-CEM (Fig. 5A) and Jurkat cells (Fig. 5B) were treated with increasing concentrations of Stattic (1.25, 2.5 and 5 μ M) or vehicle control (DMSO) for 24 h. Protein expression levels of apoptotic markers (pro-caspase-3, cleaved caspase-3) and autophagy markers (LC3B, p62) were assessed by western blotting, with β -actin used as a loading control. In CCRF-CEM cells, cleaved caspase-3 levels showed an increasing trend with higher concentrations of Stattic, indicating enhanced apoptotic activity, although the changes were not statistically significant. LC3B levels were significantly increased at 5 μ M concentrations, suggesting induction of autophagy. By contrast, the expression levels of p62, a marker of autophagic flux, remained relatively unchanged across all treatment groups, indicating incomplete autophagic flux. Pro-caspase-3 levels remained stable, further supporting that apoptosis was primarily indicated by the cleaved form (Fig. 5A). In Jurkat cells, a similar trend was observed. Cleaved caspase-3 levels increased slightly with higher Stattic concentrations, but the changes were not statistically significant. LC3B expression was significantly increased in response to 5 μ M Stattic, indicating the activation of autophagic processes. However, as in CCRF-CEM cells, p62 levels did not show a significant reduction, suggesting a potential blockade in autophagic flux. Pro-caspase-3 levels also remained constant across the different Stattic concentrations (Fig. 5B). These findings demonstrated that Stattic significantly increased autophagy markers in CCRF-CEM and Jurkat cells, while also showing a trend toward increased apoptosis in both cell lines. The differential expression patterns of LC3B and p62 suggested that Stattic may trigger autophagy but not complete autophagic degradation. The increased levels of cleaved caspase-3 suggest a potential role of Stattic in promoting apoptosis in these T-ALL cell lines, although the changes were not statistically significant.

Stattic induces early and late apoptosis in CCRF-CEM and Jurkat cells. To further confirm the pro-apoptotic effects of Stattic, flow cytometry was performed to analyze apoptosis in CCRF-CEM (Fig. 6A) and Jurkat cells (Fig. 6B) after treatment with 5 μ M Stattic for 24 h. Cells were stained with Annexin V-FITC and PI to differentiate between early and late apoptotic cells, which were detected in quadrants 3 and 2, respectively. The forward scatter (FSC)/side scatter (SSC) plots in Fig. 6A and B show the FSC and SSC characteristics of the cells, which provide information about cell size and granularity, respectively. From the results of the 5 μ M Stattic treatment, the FSC/SSC plots in CCRF-CEM (Fig. 6A) and Jurkat (Fig. 6B) cells showed an increase in the proportion of cells with reduced size, indicating cell shrinkage typically

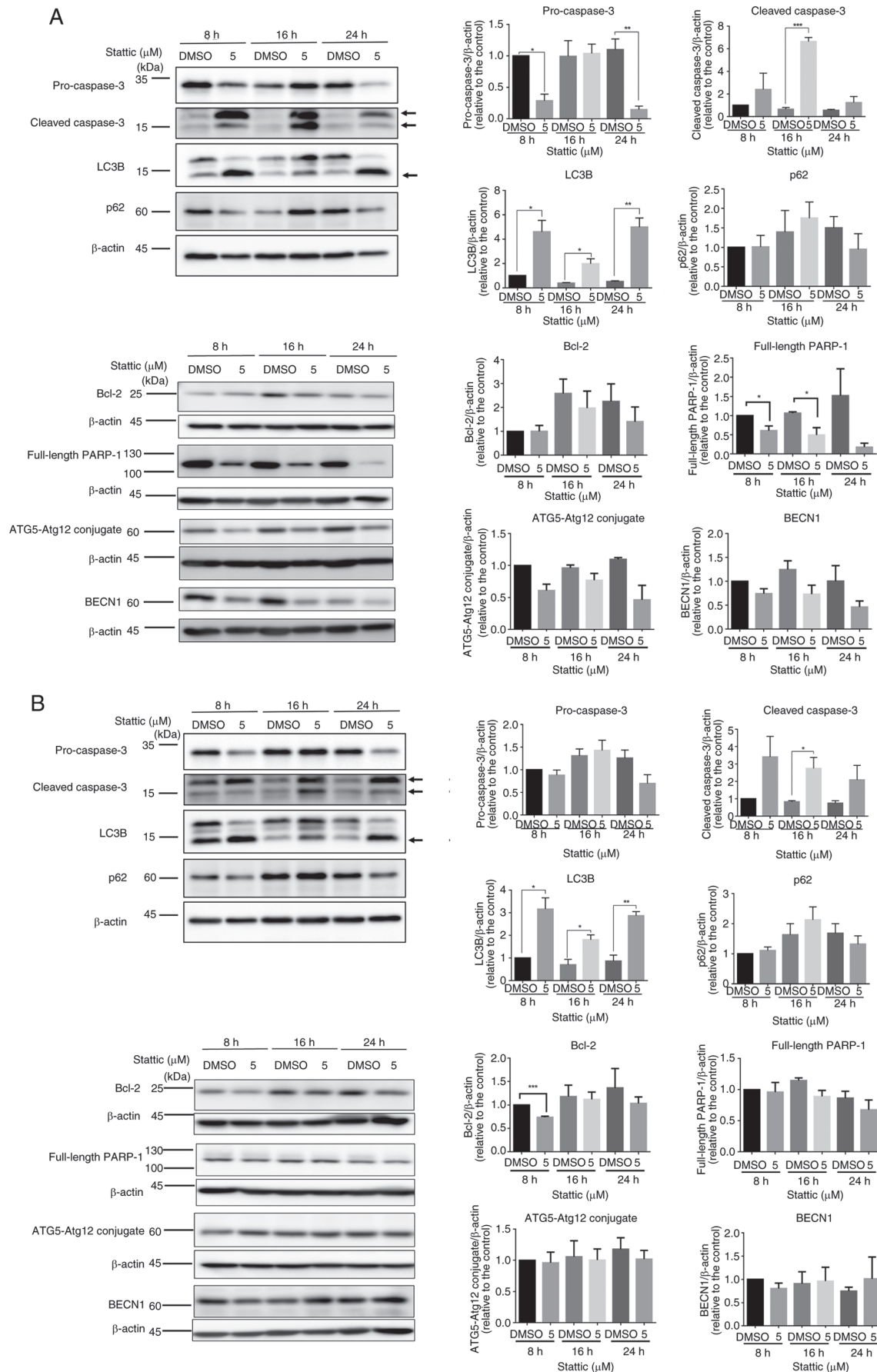


Figure 3. Stattic significantly activates cleaved caspase-3 and LC3B expression in T-cell acute lymphoblastic leukemia cells. Western blot analysis of pro-caspase-3, cleaved caspase-3, LC3B, p62, Bcl-2, PARP-1, ATG5 and BECN1 levels in (A) CCRF-CEM and (B) Jurkat cells treated with 5 μ M Stattic or DMSO for 8, 16 and 24 h. β -actin was used as a loading control. Vehicle control group was treated with 0.05% DMSO. The results are presented as the mean $\pm$ SEM (n=3). *P<0.05, **P<0.01, ***P<0.001.

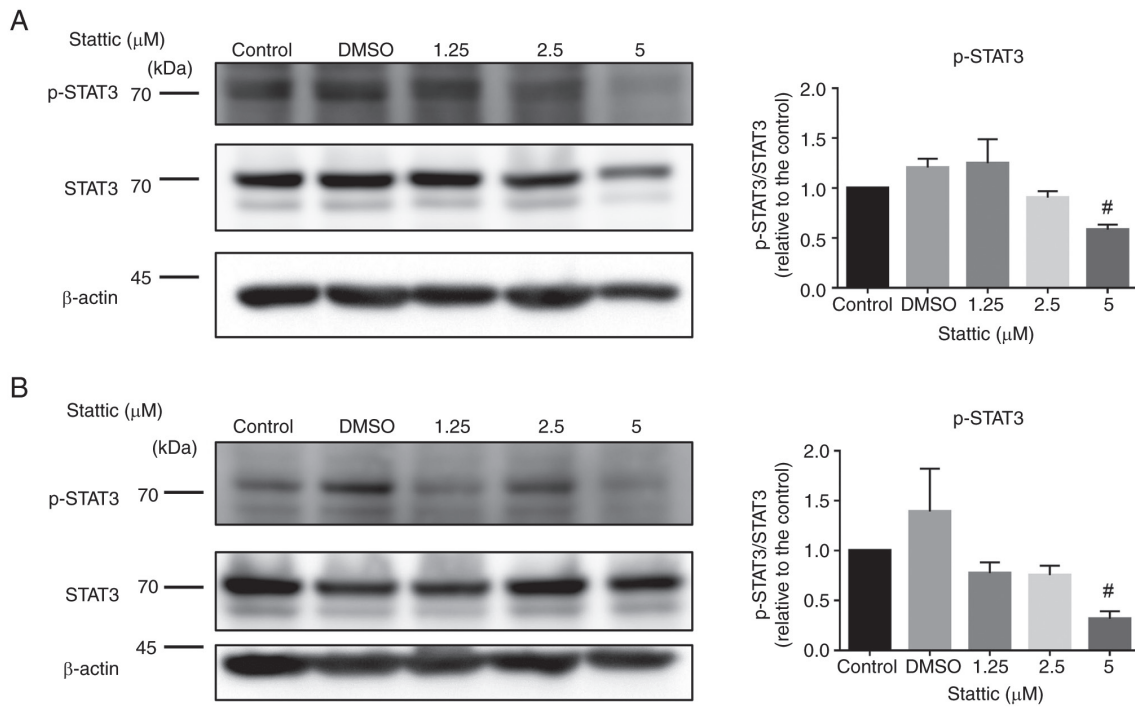


Figure 4. Stattic significantly and dose-dependently inhibits p-STAT3 expression in T-cell acute lymphoblastic leukemia cells after 24 h of treatment. Western blot analysis of p-STAT3 and total STAT3 levels in (A) CCRF-CEM and (B) Jurkat cells treated with different concentrations of Stattic (1.25, 2.5 and 5 μM) for 24 h. β-actin was used as a loading control. Vehicle control group was treated with 0.05% DMSO. The results are presented as the mean ± SEM (n=3). [#]P<0.05 vs. DMSO group. p-, phosphorylated.

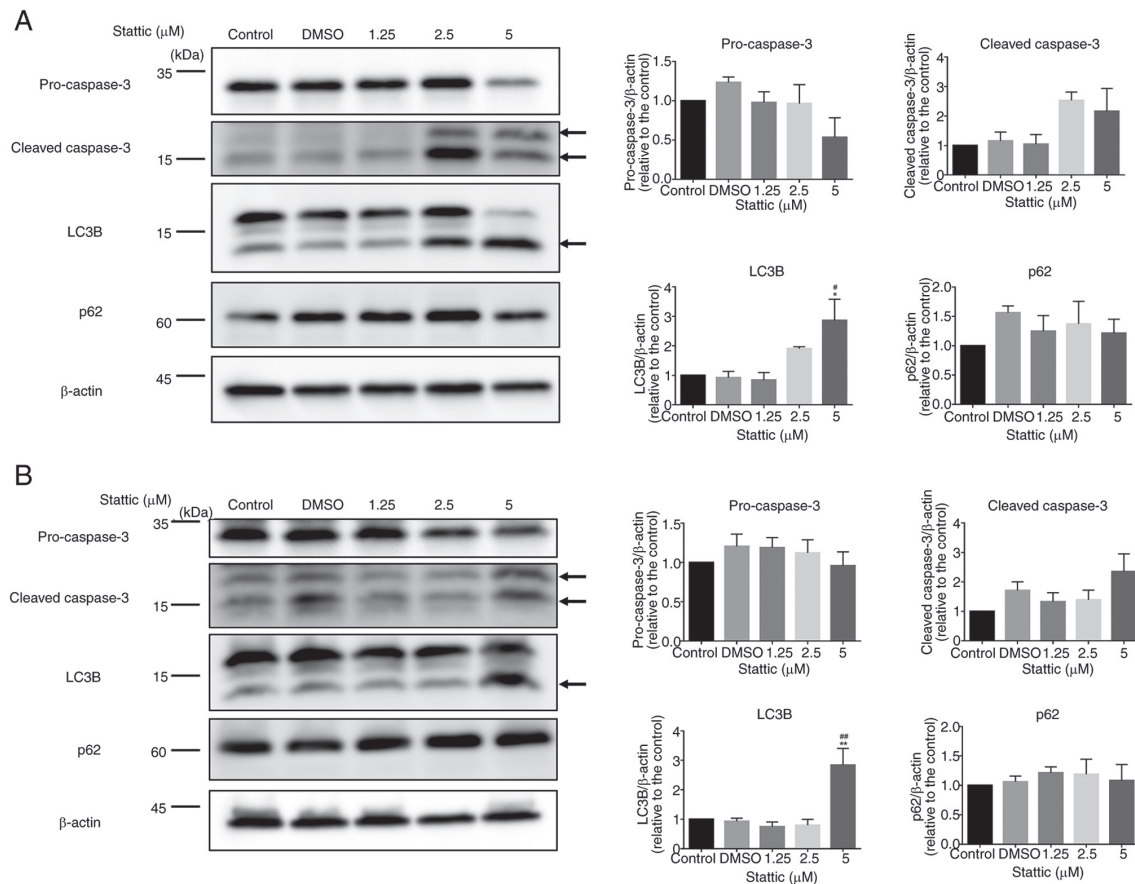


Figure 5. Stattic significantly and dose-dependently activates LC3B expression in T-cell acute lymphoblastic leukemia cells after 24 h of treatment. Western blot analysis of pro-caspase-3, cleaved caspase-3, LC3B and p62 levels in (A) CCRF-CEM and (B) Jurkat cells treated with different concentrations of Stattic (1.25, 2.5 and 5 μM) for 24 h. β-actin was used as a loading control. Vehicle control group was treated with 0.05% DMSO. The results are presented as the mean ± SEM (n=3). ^{*}P<0.05, ⁺⁺P<0.01 vs. control group; ^{*}P<0.05, ⁺⁺P<0.01 vs. DMSO group.

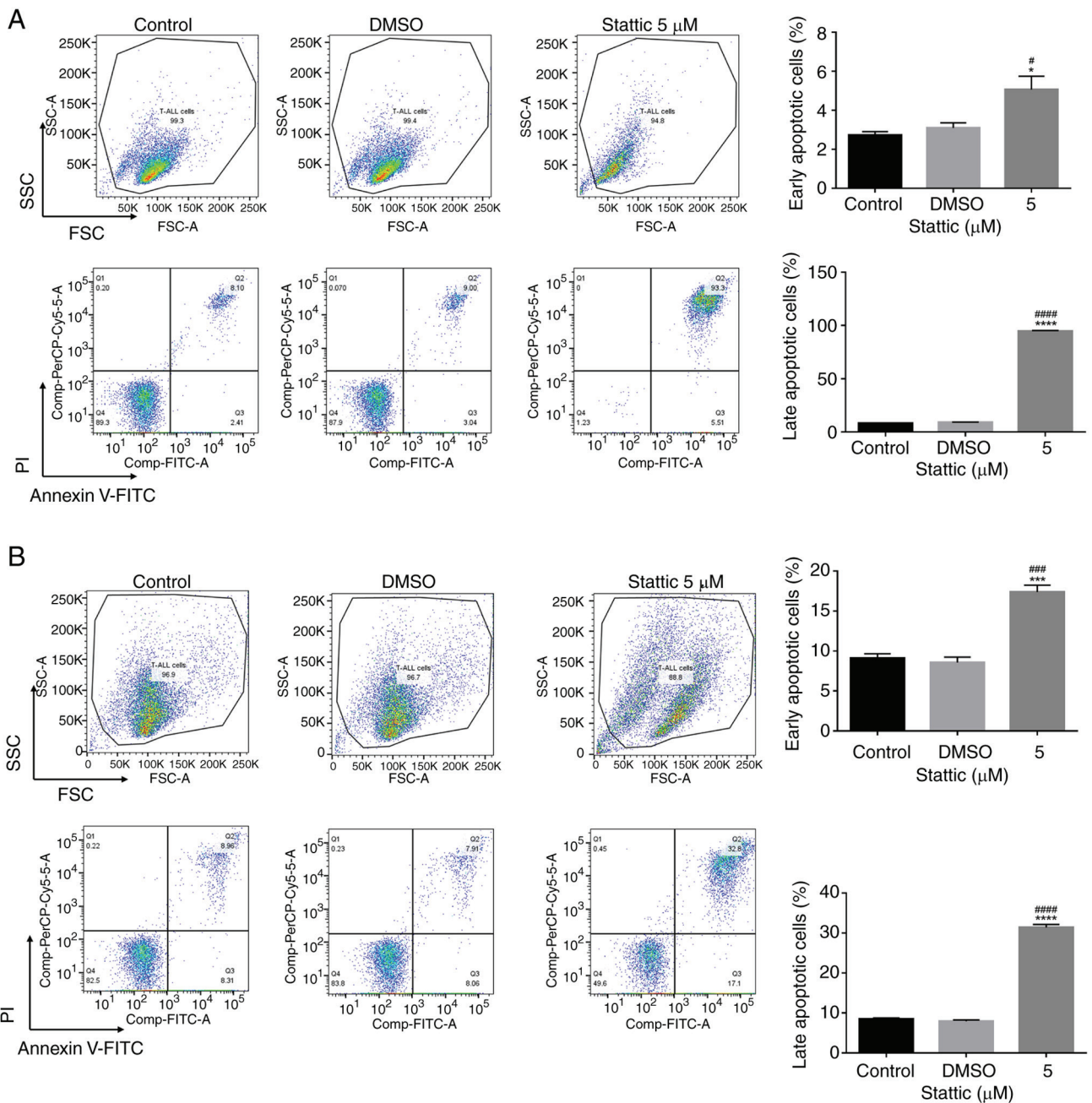


Figure 6. Stattic induces the apoptosis of T-cell acute lymphoblastic leukemia cells. (A) CCRF-CEM and (B) Jurkat cells were treated with DMSO (vehicle control) or 5 μ M Stattic for 24 h. Flow cytometric analysis of apoptosis was evaluated using Annexin V-FITC and PI double staining at 24 h post-treatment. Quantification of early and late apoptotic cells is presented as a percentage of the total cell population. Vehicle control group was treated with 0.05% DMSO. The results are presented as the mean $\pm$ SEM (n=3). *P<0.05, ***P<0.001, ****P<0.0001 vs. control group; #P<0.05, ###P<0.001, ####P<0.0001 vs. DMSO group. PI, propidium iodide.

associated with apoptosis. These results were obtained after Annexin V/PI staining, highlighting the effects of Stattic on inducing apoptotic changes in both cell lines. In CCRF-CEM cells, Stattic treatment significantly increased both early and late apoptotic populations compared with the control and DMSO groups. The percentage of early apoptotic cells increased significantly upon treatment with 5 μ M Stattic. Moreover, late apoptotic cells showed a significant increase in the Stattic-treated group, indicating that Stattic strongly induced the apoptosis of CCRF-CEM cells (Fig. 6A). Similarly, in Jurkat cells, Stattic treatment led to a significant increase in apoptosis. Early apoptotic cells were significantly

elevated, and late apoptotic cells increased substantially following treatment with 5 μ M Stattic compared with in the control and DMSO-treated groups (Fig. 6B). These results indicated that Stattic effectively promoted both early and late apoptosis in CCRF-CEM and Jurkat cells, with a particularly strong effect on late apoptosis. This supports the role of Stattic as a potent inducer of apoptosis in T-ALL cells, highlighting its therapeutic potential for T-ALL treatment.

Stattic inhibits tumor growth in the xenograft model of T-ALL. Using a xenograft mouse model of T-ALL, CCRF-CEM-xenografted mice were intraperitoneally injected

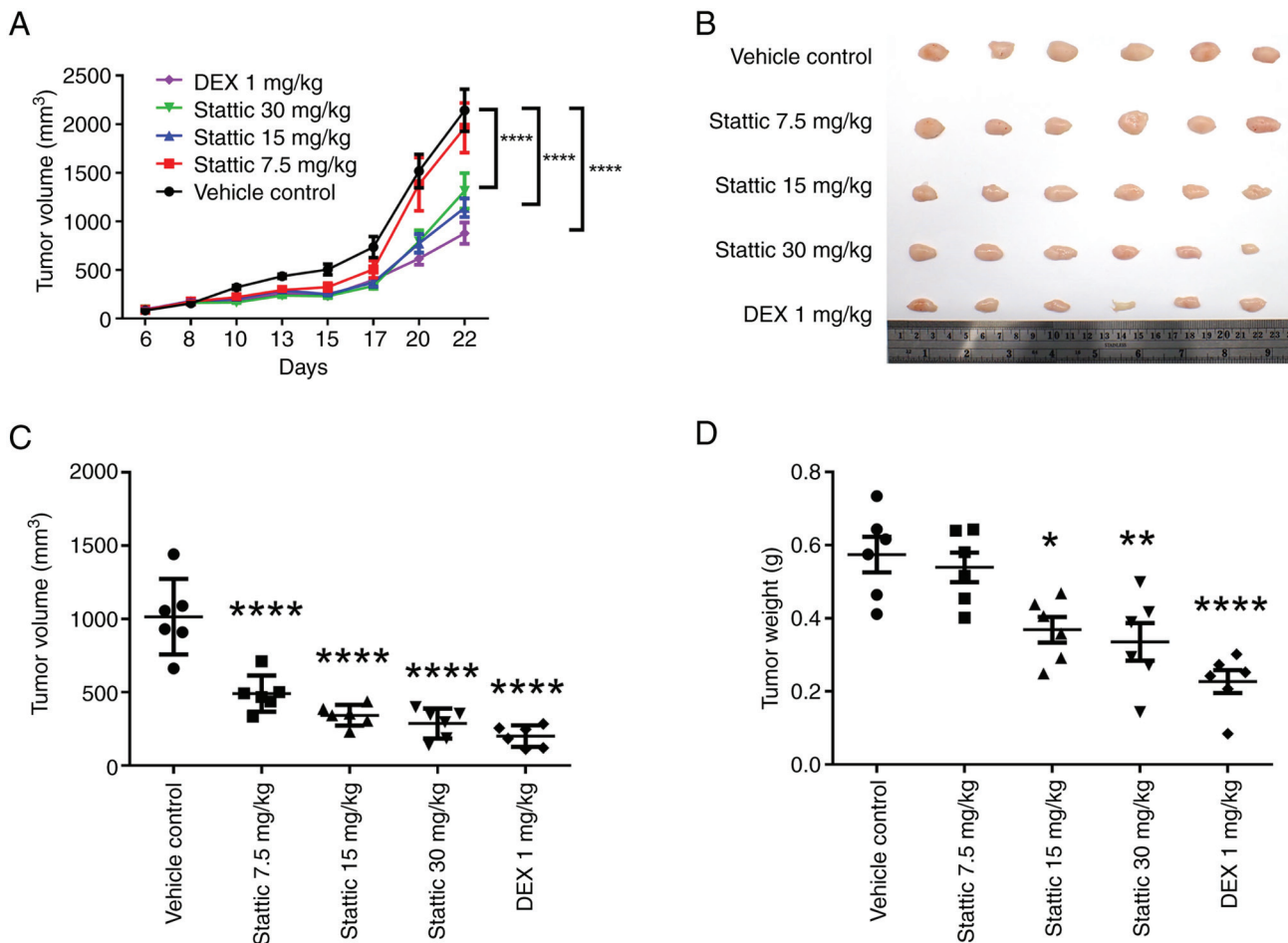


Figure 7. Efficacy of Stattic in inhibiting tumor growth in a xenograft mouse model of T-ALL. (A) Growth curves of tumor volume over time in mice treated with DEX (1 mg/kg), Stattic (7.5, 15 and 30 mg/kg) and a vehicle control (n=6/group). Tumor volumes were recorded three times weekly over a 23-day period. (B) Mice were euthanized at the end of the treatment protocol and representative images of excised tumors from each treatment group at the end of the study period are presented. (C) Tumor volumes were recorded on the last day of the experiment (day 23). (D) Tumor weights were recorded on the last day of the experiment (day 23). The results are presented as the mean $\pm$ SEM. *P<0.05, **P<0.01, ****P<0.0001, vs. vehicle control group (3% DMSO). DEX, dexamethasone.

with Stattic three times a week. The results revealed that while the control group showed a progressive growth in tumor volume over a 22-day period, the Stattic groups (15 and 30 mg/kg) and the DEX group (1 mg/kg; positive control), showed a significant reduction in tumor growth; the antitumor effect of Stattic was dose-dependent, with a peak effect observed at 30 mg/kg (Fig. 7A). Excised tumor volumes measured at the end of treatment (day 23) confirmed such a dose-dependent reduction in tumor size (Fig. 7B), and the significant decrease in volume relative to the control group in response to all doses of Stattic (Fig. 7C). In addition, a significant reduction in tumor weight was detected in the 15 and 30 mg/kg Stattic, and 1 mg/kg DEX treatment groups relative to the control (Fig. 7D). These results suggested that Stattic, along with DEX, effectively reduced tumor burden in a dose-dependent manner, with 30 mg/kg Stattic being the most effective dose.

Discussion

The present study on the effects of Stattic on T-ALL provided compelling evidence for its therapeutic potential. Notably, Stattic exhibited a dose-dependent inhibitory effect on the

viability of T-ALL cells, affirming its capacity to suppress the survival of T-ALL cells. The findings indicated that Stattic not only inhibited cell viability and p-STAT3 expression in a dose-dependent manner, but also induced cell death through apoptosis and autophagy. In addition, Stattic suppressed tumor growth in a xenograft model of T-ALL, suggesting its potential as a therapeutic agent for this malignancy.

The observed dose-dependent reduction in the viability of CCRF-CEM and Jurkat cells underscores the potent cytotoxic effects of Stattic against T-ALL cells. The findings suggested that Stattic could be effective in curtailing T-ALL progression by inhibiting cell proliferation and promoting cell death. Moreover, the significant suppression of p-STAT3 expression after Stattic treatment confirmed its action as a STAT3 inhibitor, affirming its therapeutic potential in targeting abnormal STAT3 signaling pathways in T-ALL (23). The results of the present study demonstrated that Stattic treatment may lead to a reduction in p-STAT3 levels in both CCRF-CEM cells and Jurkat cells, although the magnitude and timing of inhibition differed between the two cell lines. Specifically, CCRF-CEM cells, which have higher basal p-STAT3 levels, exhibited significant inhibition only after 24 h, whereas Jurkat

cells, with inherently lower p-STAT3 expression, displayed a similar trend but with less pronounced changes. These data suggested that the effect of Stattic on p-STAT3 is influenced by the initial expression level of p-STAT3 in the T-ALL cell line being studied. The data from both CCRF-CEM and Jurkat cells strengthen the hypothesis regarding the ability of Stattic to modulate p-STAT3-dependent pathways and provide a solid foundation for future investigations involving additional cell lines.

The present results showed a significant reduction in p-STAT3 levels only after 24 h of Stattic treatment, whereas shorter treatments (8 and 16 h) did not yield statistically significant changes. This observation is distinct from findings in some previous studies (24,25), which reported more rapid inhibition of STAT3 phosphorylation. Several factors could explain this discrepancy. First, the cell type-specific response might serve a role, as the present experiments were conducted in CCRF-CEM and Jurkat cells, which are T-ALL cell lines. These cells may exhibit a more delayed response to Stattic due to differences in the activation state of STAT3 or varying levels of basal p-STAT3 expression compared with other cell lines used in prior studies, such as solid tumor cells or other hematological malignancies. Additionally, the stability of p-STAT3 and the rate of dephosphorylation may vary among different cell lines. In some systems, STAT3 is rapidly turned over, while in others, the phosphorylation status may be sustained for longer periods. The delayed inhibition observed in the current study suggested that Stattic might require sustained exposure to accumulate sufficiently in the cells, or that a certain threshold concentration must be reached to effectively inhibit upstream kinases or disrupt STAT3 dimerization. Furthermore, experimental conditions, such as cell density, medium composition and Stattic concentration could influence the kinetics of p-STAT3 inhibition. The present study used 5 μ M Stattic, and it is possible that lower concentrations or shorter time points in previous studies led to different kinetic profiles. The delayed inhibition of p-STAT3 in the current study could reflect the need for extended Stattic exposure to overcome cellular compensatory mechanisms or gradual inhibition of signaling pathways upstream of STAT3. This might suggest that T-ALL cells are more resistant to immediate STAT3 inactivation but become vulnerable with prolonged Stattic exposure, which could be therapeutically relevant. In summary, the longer treatment duration required for significant STAT3 inhibition in the present experiments highlights the context-dependent nature of STAT3 signaling and suggests that prolonged Stattic exposure might be necessary to achieve optimal therapeutic effects in T-ALL models.

The present study also demonstrated the complex interaction between apoptosis and autophagy induced by Stattic in CCRF-CEM and Jurkat cells. Increased expression levels of both cleaved caspase-3 and LC3B markers of apoptotic and autophagic cell death suggested a dual mechanism regarding the promotion of cell death by Stattic. The dose-dependent nature of these responses further highlighted the ability of Stattic to effectively modulate these key cell death pathways (26).

The translational significance of the present results is supported by the *in vivo* efficacy of Stattic in reducing tumor growth in a xenograft mouse model of T-ALL. Stattic led to a dose-dependent decrease in tumor growth, with the highest

dose generating the greatest antitumor effect. Targeting STAT3 is known to inhibit tumor growth in various types of cancer, such as colorectal cancer (27), breast cancer (28) and glioma (29). These findings corroborate the present *in vitro* data, and highlight the potential of Stattic as a targeted therapeutic for T-ALL. These *in vivo* results further indicated the translational potential of Stattic, predicting its move into clinical trial phase. Such an approach provides a new option for T-ALL therapy that targets STAT3 signaling, a critical pathway in the pathogenesis of numerous malignancies.

The primary limitations of the present study include the reliance on the CCRF-CEM and Jurkat cell lines, and a xenograft mouse model, which might not fully capture the biological complexity and heterogeneity of human T-ALL. The short-term nature of these experiments cannot reflect long-term outcomes or potential resistance mechanisms to Stattic treatment. Further research is needed to fully understand the therapeutic potential and limitations of Stattic in treating T-ALL. Extending studies to include diverse T-ALL subtypes, long-term treatment effects and comprehensive safety profiles would provide more robust data to support clinical applications, and strengthen the preliminary findings of the current study. As part of our future studies, we plan to use CRISPR-Cas9 or RNA interference approaches to knock out or knock down STAT3 expression in CCRF-CEM cells and compare the resulting effects with those of Stattic treatment. This additional work will provide more direct evidence of STAT3 dependency.

In conclusion, the results of the present study support the potential of Stattic as a therapeutic agent against T-ALL by inhibiting STAT3 signaling, and inducing programmed cell death through apoptosis and autophagy. The present findings may pave the way for further clinical investigations into Stattic and emphasize the importance of targeting dysregulated STAT3 signaling in leukemia therapy.

Acknowledgements

Not applicable.

Funding

This work was supported by grants from the Tri-Service General Hospital Penghu Branch, Taiwan (grant no. TSGH-PH_D_112002) and Taichung Veterans General Hospital, Taiwan (grant nos. TCVGH-1136505C, TCVGH-HK1138001 and TCVGH-YM1130108). This project was also supported by grants from the National Science and Technology Council, Taiwan (grant nos. NSTC 112-2914-I-075A-002-A1 and NSTC 111-2314-B-075A-009).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CLL, HYC and FLH conceptualized the study. CLL, HYC, JCY and SJY designed the methodology. CLL, TYC and SWY performed the formal analysis. CLL, HYC, JCY and SJY

conducted the investigation. CLL prepared the original draft, while CLL, HYC and FLH reviewed and edited the manuscript. CLL, HYC and FLH managed the project. HYC, JCY, HYC and FLH secured funding. CLL, SJY and FLH confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experimental protocols were approved by the Animal Care and Use Committee of the Taichung Veterans General Hospital (IACUC no. La-1132052).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Inaba H, Greaves M and Mullighan CG: Acute lymphoblastic leukaemia. *Lancet* 381: 1943-1955, 2013.
- Puumala SE, Ross JA, Aplenc R and Spector LG: Epidemiology of childhood acute myeloid leukemia. *Pediatric Blood Cancer* 60: 728-733, 2013.
- Zhang N, Wen D, Wang T and Deng J: Disparities in incidence and mortality of pediatric acute lymphoblastic leukemia across countries with different incomes. *Leukemia*: October 4, 2024 (Epub ahead of print).
- Lupo PJ and Spector LG: Cancer progress and priorities: Childhood cancer. *Cancer epidemiology, biomarkers & prevention: A publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 29: 1081-1094, 2020.
- Faderl S, O'Brien S, Pui CH, Stock W, Wetzler M, Hoelzer D and Kantarjian HM: Adult acute lymphoblastic leukemia: Concepts and strategies. *Cancer* 116: 1165-1176, 2010.
- Redaelli A, Laskin BL, Stephens JM, Botteman MF and Pashos CL: A systematic literature review of the clinical and epidemiological burden of acute lymphoblastic leukaemia (ALL). *Eur J Cancer Care (Engl)* 14: 53-62, 2005.
- Tzoneva G, Perez-Garcia A, Carpenter Z, Khiabanian H, Tosello V, Allegretta M, Paietta E, Racevskis J, Rowe JM, Tallman MS, *et al*: Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. *Nat Med* 19: 368-371, 2013.
- Fielding AK, Richards SM, Chopra R, Lazarus HM, Litzow MR, Buck G, Durrant IJ, Luger SM, Marks DI, Franklin IM, *et al*: Outcome of 609 adults after relapse of acute lymphoblastic leukemia (ALL): an MRC UKALL12/ECOG 2993 study. *Blood* 109: 944-950, 2007.
- Sas V, Moisoiu V, Teodorescu P, Tranca S, Pop L, Iluta S, Pasca S, Blag C, Man S, Roman A, *et al*: Approach to the adult acute lymphoblastic leukemia patient. *J Clin Med* 8: 1175, 2019.
- Bansal M, Sharma KK, Bakhshi S and Vatsa M: Perception of Indian parents on health-related quality of life of children during maintenance therapy of acute lymphoblastic leukemia: A comparison with siblings and healthy children. *J Pediatr Hematol Oncol* 36: 30-36, 2014.
- Reinfjell T, Lofstad GE, Nordahl HM, Vikan A and Diseth TH: Children in remission from acute lymphoblastic leukaemia: Mental health, psychosocial adjustment and parental functioning. *Eur J Cancer Care (Engl)* 18: 364-370, 2009.
- Khalifa AS, Bishry Z, Tantawy AAG, Ghanem MH, Effat SM, Shahawy HE and Ebeid FSE: Psychiatric morbidity in Egyptian children with acute lymphoblastic leukemia and their care providers. *Hematol Oncol Stem Cell Ther* 7: 76-84, 2014.
- Kantarjian H, Thomas D, O'Brien S, Cortes J, Giles F, Jeha S, Bueso-Ramos CE, Pierce S, Shan J, Koller C, *et al*: Long-term follow-up results of hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (Hyper-CVAD), a dose-intensive regimen, in adult acute lymphocytic leukemia. *Cancer* 101: 2788-2801, 2004.
- Mantadakis E, Cole PD and Kamen BA: High-dose methotrexate in acute lymphoblastic leukemia: Where is the evidence for its continued use? *Pharmacotherapy* 25: 748-755, 2005.
- Schmiegelow K, Nielsen SN, Frandsen TL and Nersting J: Mercaptopurine/Methotrexate maintenance therapy of childhood acute lymphoblastic leukemia: Clinical facts and fiction. *J Pediatr Hematol Oncol* 36: 503-517, 2014.
- Burnett AK, Milligan D, Prentice AG, Goldstone AH, McMullin MF, Hills RK and Wheatley K: A comparison of low-dose cytarabine and hydroxyurea with or without all-trans retinoic acid for acute myeloid leukemia and high-risk myelodysplastic syndrome in patients not considered fit for intensive treatment. *Cancer* 109: 1114-1124, 2007.
- Huguet F and Tavtavian S: Emerging biological therapies to treat acute lymphoblastic leukemia. *Expert Opin Emerg Drugs* 22: 107-121, 2017.
- Pan Y, Zhou F, Zhang R and Claret FX: Stat3 inhibitor stattic exhibits potent antitumor activity and induces chemo- and radio-sensitivity in nasopharyngeal carcinoma. *PLoS One* 8: e54565, 2013.
- Buettner R, Mora LB and Jove R: Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin Cancer Res* 8: 945-954, 2002.
- Zhang Q, Zhang C, He J, Guo Q, Hu D, Yang X, Wang J, Kang Y, She R, Wang Z, *et al*: STAT3 inhibitor stattic enhances radiosensitivity in esophageal squamous cell carcinoma. *Tumour Biol* 36: 2135-2142, 2015.
- Schust J, Sperl B, Hollis A, Mayer TU and Berg T: Stattic: A small-molecule inhibitor of STAT3 activation and dimerization. *Chem Biol* 13: 1235-1242, 2006.
- Lin L, Liu A, Peng Z, Lin HJ, Li PK, Li C and Lin J: STAT3 is necessary for proliferation and survival in colon cancer-initiating cells. *Cancer Res* 71: 7226-7237, 2011.
- Kanna R, Choudhary G, Ramachandra N, Steidl U, Verma A and Shastri A: STAT3 inhibition as a therapeutic strategy for leukemia. *Leuk Lymphoma* 59: 2068-2074, 2018.
- Li CH, Xu LL, Jian LL, Yu RH, Zhao JX, Sun L, Du GH and Liu XY: Stattic inhibits RANKL-mediated osteoclastogenesis by suppressing activation of STAT3 and NF- κ B pathways. *Int Immunopharmacol* 58: 136-144, 2018.
- Mikyskova R, Sapega O, Psotka M, Novotny O, Hodny Z, Balintova S, Malinak D, Svobodova J, Andrys R, Rysanek D, *et al*: STAT3 inhibitor Stattic and its analogues inhibit STAT3 phosphorylation and modulate cytokine secretion in senescent tumour cells. *Mol Med Rep* 27: 81, 2023.
- Debnath J, Gammoh N and Ryan KM: Autophagy and autophagy-related pathways in cancer. *Nat Rev Mol Cell Biol* 24: 560-575, 2023.
- Huang L, Zhao Y, Shan M, Wang S, Chen J, Liu Z and Xu Q: Targeting crosstalk of STAT3 between tumor-associated M2 macrophages and Tregs in colorectal cancer. *Cancer Biol Ther* 24: 2226418, 2023.
- Yue P, Zhu Y, Brotherton-Pleiss C, Fu W, Verma N, Chen J, Nakamura K, Chen W, Chen Y, Alonso-Valenteen F, *et al*: Novel potent azetidine-based compounds irreversibly inhibit Stat3 activation and induce antitumor response against human breast tumor growth in vivo. *Cancer Lett* 534: 215613, 2022.
- Zhang L, Alizadeh D, Van Handel M, Kortylewski M, Yu H and Badie B: Stat3 inhibition activates tumor macrophages and abrogates glioma growth in mice. *Glia* 57: 1458-1467, 2009.



Copyright © 2024 Li et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.