Paclitaxel mediates the PI3K/AKT/mTOR pathway to reduce proliferation of FLT3-ITD$^+$ AML cells and promote apoptosis

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Abstract. Acute myeloid leukemia (AML) with internal tandem duplication (ITD) mutations in the FLT3 tyrosine kinase tend to have a poor prognosis. FLT3-ITD can promote the progress of AML by activating the PI3K/AKT/mTOR pathway. Paclitaxel (PTX) is a natural anticancer drug that has been widely used in chemotherapy for multiple malignancies. The present study used the CCK-8 assay, flow cytometry, PCR and western blotting to explore the anti-leukemia effect and possible mechanisms of PTX on MV4-11 cells with the FLT3-ITD mutation and the underlying mechanism. As a result, it was found that PTX could inhibit proliferation of MV4-11 cells and promoted apoptosis by inhibiting the PI3K/AKT/mTOR pathway.

Introduction

Acute myeloid leukemia (AML) is a common malignancy of the blood system and has characteristics of genetic diversity, aggression and high heterogeneity (1). Most AML patients have gene mutations associated with the occurrence, development and prognosis of leukemia (2-5), including the internal tandem duplication (ITD) mutations of the FLT3 tyrosine kinase (FLT3-ITD) gene. Of AML patients ~30% carry these mutations (6,7) with adverse effects on treatment outcomes (8,9). The FLT3-ITD mutation causes autophosphorylation of the FLT3 receptor in the absence of its ligand (10), resulting in activation of downstream PI3K/AKT, RAS/MAPK/ERK and STAT5 signaling pathways (11,12). Ultimately, proliferation and survival of leukemia cells are both enhanced (13-15). Therefore, the FLT3-ITD has been considered a therapeutic target (16) and several FLT3 inhibitors explored (4). However, inhibitory effects are limited (17,18) by acquired resistance caused by a secondary point mutation in the activation loop of the FLT3, producing FLT3-TKD, which often causes patients to relapse after remission (16,19,20). Thus, the search continues for new drugs and treatment strategies for AML patients.

The anti-tumor drug, paclitaxel (PTX) (21,22), promotes tubulin aggregation, stabilizes microtubule structure, inhibits cell mitosis and arrests cells in the G_2/M phase of the cell cycle (23). The drug also regulates Bcl-2 and Caspase-3 to promote apoptosis of cancer cells (24,25). Increasing evidence suggests that PTX mediates the PI3K/AKT signaling pathway to inhibit proliferation of solid tumor cells and induce apoptosis. Such actions have been reported for nasopharyngeal carcinoma (26), cervical cancer (27) and lung cancer (28). Paclitaxel also enhances the sensitivity of cancer cells to other antitumor drugs through regulation of this pathway (28,29). Inhibitory effects have also been found for PTX with leukemia cell lines, such as HL-60 (30) and K562 (31,32). Our previous study (33) showed that PTX combined with quizartinib synergistically inhibited proliferation and induced apoptosis of the FLT3-ITD$^+$ AML cell-line, MV4-11, but how PTX acts on the MV4-11 cells remains to be elucidated.

The current study sought to clarify the effect of PTX on proliferation and apoptosis of MV4-11 cells and to investigate the underlying mechanism. Paclitaxel was found to have anti-proliferative and apoptosis-inducing effects on MV4-11 cells via an underlying mechanism connected with the PI3K/AKT/mTOR pathway.

Materials and methods

Cell culture. MV4-11 (FLT3-ITD$^+$ AML cell-line) cells were obtained from Nanjing Kebai Biotechnology Co., Ltd. and THP-1 and K562 (FLT3-ITD$^-$ leukemia cell-lines) cells from Guangzhou Saiku Biotechnology Co., Ltd. and Procell Life Science & Technology Co., Ltd., respectively. MV4-11 cells were cultured in Iscove's Modified Dulbecco's Medium (Corning, Inc.) and THP-1 and K562 cells in RPMI-1640 medium (Corning, Inc.) both containing penicillin-streptomycin solution and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) 37˚C, 5% CO_2 in a humidified incubator.

Reagents. Paclitaxel and sorafenib were obtained from Shanghai Yuanye Biotechnology Co., Ltd. and PKI-587 from

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Shanghai Macklin Biochemical Co., Ltd. Cell Counting Kit-8 (CCK8) was from Dojindo Laboratories, Inc. and Annexin V-FLTC/propidium iodide (PI) Apoptosis Assay Kit (cat. no. 56584) from BD Biosciences. Reverse transcription and PCR kits were from Takara Biotechnology Co., Ltd. Anti-FLT3 antibody (1:1,000; cat. no. 3462), anti-AKT antibody (1:1,000; cat. no. 4691), anti-phosphorylated (p-)AKT antibody (1:2,000; cat. no. 4060), anti-S6K antibody (1:1,000; cat. no. 2708) and anti-p- S6K antibody (1:1,000; cat. no. 9234) were all purchased from Cell Signaling Technology, Inc. and anti-GAPDH antibody (1:5,000; cat. no. 10494-1-AP) was from Proteintech Group, Inc. HRP-conjugated secondary antibodies goat anti-rabbit (1:2,000; cat. no. M21002S) was purchased from Abmart Pharmaceutical Technology Co., Ltd.

CCK8 assay. MV4-11, THP-1 and K562 cells were seeded into 96-well plates and 10 μl per well CCK-8 added at time-points indicated for 1-4 h before the absorbance (OD) value was measured at 450 nm by microplate reader. Mean OD values at each concentration were used to calculate the proliferation inhibition rate=[1-(experimental group OD value-blank group OD value)/(negative control group OD value-blank group OD value)] x100%. Experiments were performed in triplicate. Half-maximal inhibitory concentration (IC_{50}) values were calculated by GraphPad Prism version 5.0 software (Dotmatics).

Effects of Paclitaxel plus PKI-587 on proliferation of MV4-11 cells. The synergistic index, q, was calculated using the Kingsdale formula (34) to assess the combined effect of the two drugs. The formula was: $q=E(D_{1+2})/(D_1+D_2-D_1xD_2)$, where D_1 and D_2 are individual rates of inhibition by the two drugs and D_{1+2} is the combined rate of inhibition. Values of $q>1.15$, $0.85\leq q\leq 1.15$ and $q<0.85$ indicate synergistic, additive and antagonistic effects, respectively.

Flow cytometry assay. Aliquots of $1x10^6$ cells/ml of MV4-11, THP-1 and K562 cells were treated for 48 h before harvesting and washing once with PBS precooled at 4˚C. Cells were resuspended with 1X Binding Buffer and 5 μl Annexin V added with incubation at room temperature for 15 min in the dark. 5 μl PI and 300 μl 1X Binding Buffer were added, at room temperature for 5 min. FACSCalibur (BD Biosciences) was used to perform flow cytometry within 5 min. FlowJo 10.0.7 software (FlowJo LLC) was used for analysis. Apoptotic rate=the percentage of early + late apoptotic cells.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted by TRIzol® reagent (Thermo Fisher Scientific, Inc.) from $1x10^6$ cells/ml of MV4-11 cells which had been exposed to various concentrations of PTX for 48 h. Samples were dried and RNA dissolved in DEPC water. The concentration was adjusted to give an OD_{260}/OD_{280} ratio between 1.8-2.0 and Prime Script RT Master Mix reverse transcription kit used to perform the reverse transcription into DNA at 37˚C for 15 min followed by inactivation at 85˚C for 5 min, according to the manufacturer's protocol. RT-qPCR was performed using TB Green Premix Ex Taq II kit with GAPDH as internal reference, according to the manufacturer's instructions. Fold change in expression levels was calculated using the $2^{-\Delta\Delta Cq}$ method (35). The Fluorescent PCR instrument, Bio-Rad CFX Manager 2.1 was purchased from Bio-Rad Laboratories, Inc. All RT-qPCR primers were designed by Sangon Biotech Co., Ltd. and the primer sequences were as follows: FLT3, forward, 5'-GCA ATCATAAGCACCAGCCAGGA-3' and reverse, 5'-TTCTGC GAGCACTTGAGGTTTCC-3'; PI3K, forward, 5'-CTTTGC GACAAGACTGCCGAGAG-3' and reverse, 5'-CGCCTG AAGCTGAGCAACATCC-3'; AKT, forward, 5'-ATGGAG TATGCCAACGGGGG-3' and reverse, 5'-TGTCGCGGTATA CCACGTC-3'; mTOR, forward, 5'-CTTGCTGAACTGGAG GCTGATGG-3' and reverse, 5'-CCGTTTTCTTATGGGCTG GCTCT-3'; S6K, forward, 5'-TGCTGTGGATTGGTGGAG TTTGG-3' and reverse, 5'-TCTGGCTTCTTGTGTGAGGTA GGG-3'; GAPDH, forward, 5'-CTCTGCTCCTCCTGTTCG AC-3' and reverse, 5'-TAAAAGCAGCCCTGGTGAC-3'. The thermocycling conditions were: Initial denaturation at 95˚C for 30 sec, denaturation at 95˚C for 5 sec, annealing and elongation at 60˚C for 30 sec for a total of 40 cycles.

Western blotting. Total protein was extracted from $1x10^6$ cells/ml MV4-11 cells which had been exposed to various concentration of PTX for 48 h. In brief, cells were washed twice with pre-cooled PBS and radioimmunoprecipitation assay lysis buffer (RIPA; Beyotime Institute of Biotechnology) added before protein concentrations were determined by Enhanced Bicinchoninic acid Protein Assay Kit (BCA; Beyotime Institute of Biotechnology). Protein samples (10 μl per lane) were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to PVDF membrane (Millipore, USA). Membranes were blocked for 1 h at room temperature with 5% BSA blocking buffer (Beijing Solarbio Science & Technology Co., Ltd.) and incubated with primary antibodies overnight at 4˚C. Membranes were incubated with secondary antibodies for 1 h at room temperature and ECL luminescence substrate kit (Biosharp Life Sciences) and ImageJ version 1.48 software (National Institutes of Health) were used to visualize and quantify protein bands.

Statistical analysis. SPSS17.0 software (SPSS, Inc.) was used to perform all statistical analyses. Data are presented as mean ± standard deviation. When groups=3, differences were analyzed by one-way ANOVA with the Least-Significant Difference (LSD) for post hoc multiple comparisons. When groups >3, differences were analyzed by one-way ANOVA with Tukey's for post hoc multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of PTX on proliferation of leukemia cells. To assess the effect of PTX on the FLT3-ITD+ AML cells, MV4-11, the present study first confirmed its effect on cell viability and compared it with that of FLT3 inhibitor sorafenib, which has been widely used in treatment of AML patients. It used various concentrations of PTX and sorafenib to treat MV4-11 cells for 24, 48 and 72 h. The results showed that PTX inhibited proliferation of MV4-11 cells in a dose-dependent manner and with increased potency with prolonged exposure (Fig. 1A).

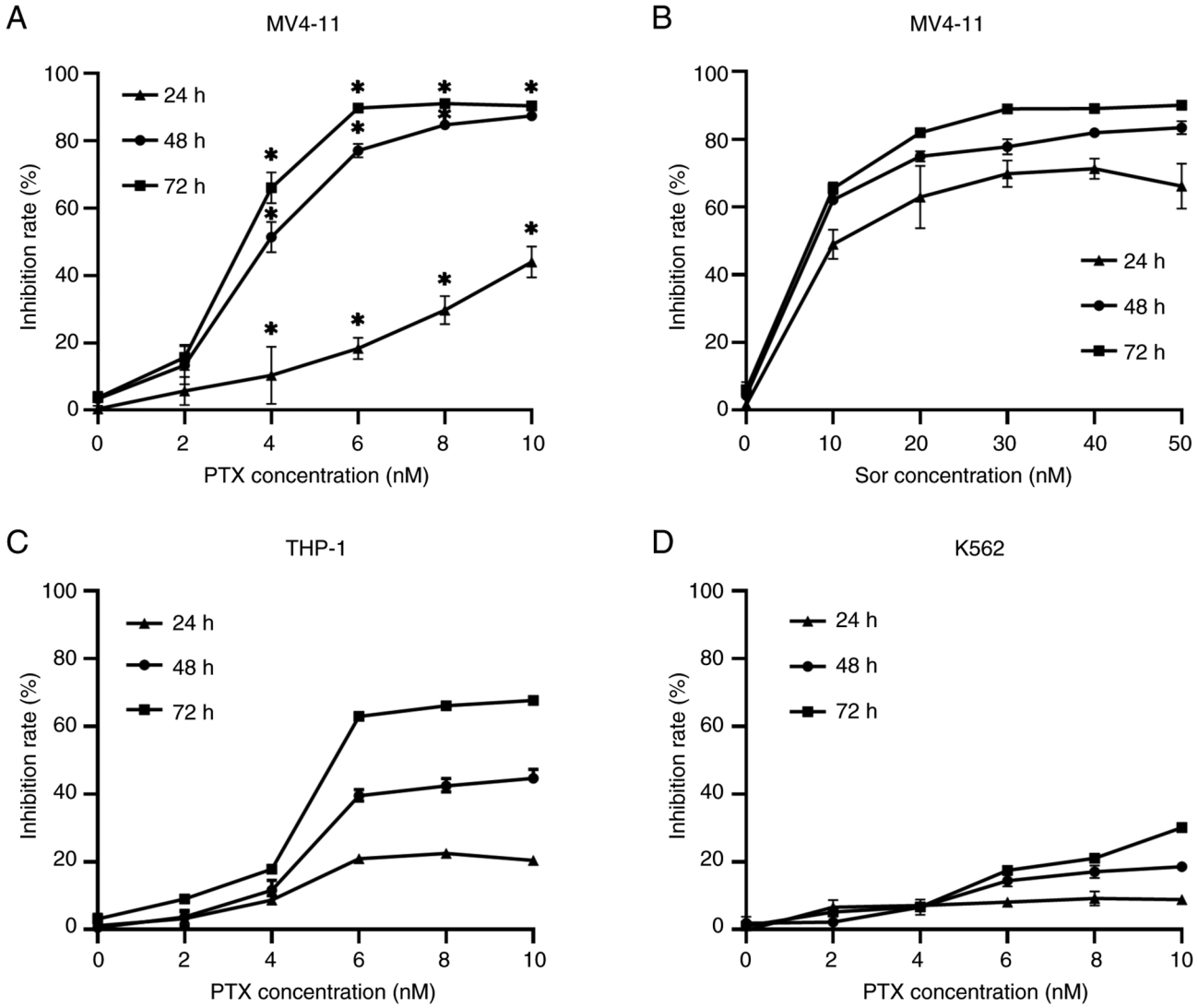

Figure 1. Effect of PTX on proliferation of leukemia cells. Various concentrations of paclitaxel were used to treat (A) MV4-11, (C) THP-1 and (D) K562 cells for 24, 48 and 72 h. (B) Various concentrations of Sor were used to treat MV4-11 cells for 24, 48 and 72 h. Cell growth was measured by CCK8 assay. *P<0.05. PTX, paclitaxel; Sor, sorafenib.

Sorafenib also had a significant inhibitory effect on cell proliferation (Fig. 1B). By comparing the IC_{50} values of both drugs for inhibition of MV4-11 cell proliferation, it was found that the inhibitory effect of PTX was stronger than that of sorafenib (Table I). To further test and compare the anti-proliferative effect of PTX on the FLT3-ITD$^-$ leukemia cells, THP-1 and K562, the present study used the same concentrations of PTX to treat THP-1 and K562 cells, respectively. It was observed that there was a more moderate inhibitory effect on proliferation (Fig. 1C and D) and PTX IC_{50} values for inhibition of THP-1 and K562 cell proliferation were larger than that for MV4-11 cells (Table I). These results indicated that PTX could clearly inhibit proliferation of MV4-11 cells.

Effect of PTX on apoptosis in leukemia cells. MV4-11, THP-1 and K562 cells were treated with a range of PTX concentrations for 48 h before evaluation of apoptosis by flow cytometry. Rates of apoptosis elicited by PTX in the different cell-lines were: MV4-11, 10 nM PTX; 93.53±0.31% and 20 nM PTX: 95.37±0.67%; THP-1 cells, 10 nM PTX: 7.31±0.23% and 20 nM PTX: 53.23±0.9%; K562 cells, 10 nM PTX: 11.67±0.43% and 20n M PTX: 12.68±0.58%. PTX induced apoptosis in leukemia cells at higher rates for MV4-11 cells compared with THP-1 and K562 cells (Fig. 2). Then, in order to compare the pro-apoptotic effects of PTX and sorafenib on MV4-11 cells, the same concentration of PTX and sorafenib was used to treat MV4-11 cells respectively for 48 h. It was found that the apoptosis rate of sorafenib at 4 nM was slightly higher than that of PTX, but at 8 nM, the apoptosis rate of PTX, 91.2±0.82%, was significantly higher compared with that of sorafenib, 48.03±0.15% (Fig. 3). Therefore, it was hypothesized that the pro-apoptotic effect of PTX on MV4-11 cells was stronger than sorafenib.

Effect of PTX on expression of FLT3 mRNA and protein in MV4-11 cells. Given that PTX reduced proliferation and promoted apoptosis in MV4-11 cells, the underlying mechanism was investigated by treating MV4-11 cells with 10 or 20 nM PTX for 48 h, followed by PCR and western blotting. FLT3 mRNA (Fig. 4A) and protein (Fig. 4B and C)

Table I. IC_{50} values for growth rate inhibition by paclitaxel and sorafenib for leukemia cells.

Drug action duration	Paclitaxel (nM)			Sorafenib (nM)
	MV4-11	THP-1	K562	MV4-11
24 h	14.35±1.22	35.84±8.77	99.29±2.59[a]	16.84±1.41
48 h	5.65±0.35	11.53±1[b]	33.13±3.85[b]	11.41±0.9[c]
72 h	3.92±0.18	7.01±0.12[a]	17.65±0.95[a]	8.95±1.67[c]

Various concentrations of paclitaxel were used to treat MV4-11, THP-1 or K562 cells and sorafenib was used to treat MV4-11 for 24, 48 and 72 h. Cell growth was measured by CCK8 assay and the IC_{50} values were calculated by GraphPad Prism Version 5.0 software. Data is presented as mean ± standard deviation (n=3). At the same time of action, vs. MV4-11 cells, [a]P<0.01, [b]P<0.001. At the same time of action, for MV4-11cells, vs. paclitaxel, [c]P<0.05. IC_{50}, half-maximal inhibitory concentration.

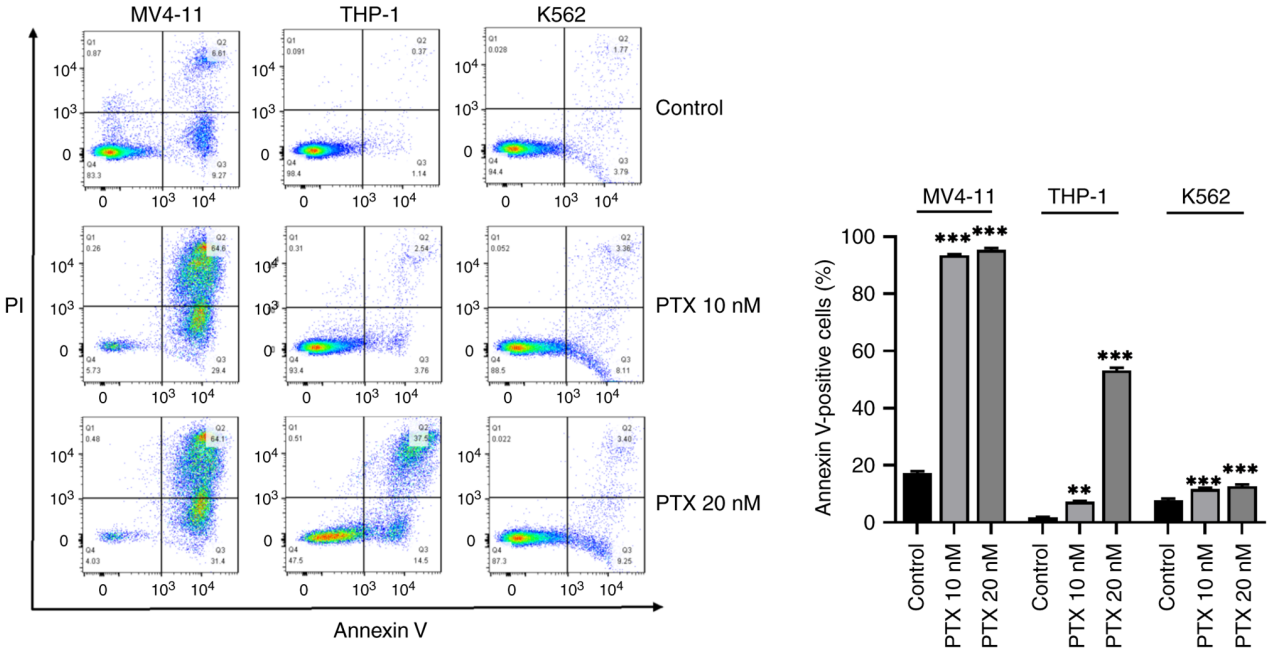

Figure 2. Effect of PTX on apoptosis in leukemia cells. MV4-11 cells, THP-1 cells and K562 cells were treated with 10 or 20 nM PTX for 48 h, followed by flow cytometry assay. **P<0.01, ***P<0.001. PTX, paclitaxel.

expression were significantly downregulated compared with control cells and with the increase of PTX concentration, FLT3 gene expression decreased significantly. Thus, downregulation of the FLT3 gene may be linked to decreased growth of MV4-11 cells.

Effect of PTX on PI3K/AKT/mTOR pathway in MV4-11 cells. The FLT3 receptor activates the downstream PI3K/AKT/mTOR signaling pathway which is known to be involved in growth of leukemia cells (36). According to the results of the previous experiment, PTX can inhibit the expression of FLT3 gene so, to test whether PTX have any effect on the PI3K/AKT/mTOR pathway, MV4-11 cells were treated with 0, 10 or 20 nM PTX for 48 h and expression of genes dependent on PI3K/AKT/mTOR signaling measured by PCR and western blotting. Production of PI3K, AKT, mTOR and S6K mRNA were all downregulated compared with the control cells (Fig. 5). Compared with the control group, the expression of p-AKT (Fig. 6A) and p-S6K (Fig. 6B) protein were also decreased significantly, but the changes of AKT, S6K protein were not evident (Fig. 6B). Based on this experiment, it was hypothesized that PTX might mainly inhibit the phosphorylation of AKT and S6K. Thus, the inhibitory effect of PTX on MV4-11 cell growth may be linked to inhibition of the PI3K/AKT/mTOR pathway.

Effect of the PI3K/AKT/mTOR pathway inhibitor, PKI-587, in inhibiting growth of MV4-11 cells. The above experiments showed that PTX could not only have anti-proliferative and pro-apoptotic effect on MV4-11 cells, but also inhibited PI3K/AKT/mTOR pathway in the cells. To test whether the effect of PTX on MV4-11 cells depend on this signaling pathway, cells were treated with PI3K/AKT/mTOR pathway inhibitor PKI-587, which also known as gedatolisib or PF 05212384. PKI-587 could inhibit cell viability (Fig. 7). Taken together, it was hypothesized that the inhibitory effect of PTX on MV4-11 cells proliferation was related to the PI3K/AKT/mTOR pathway.

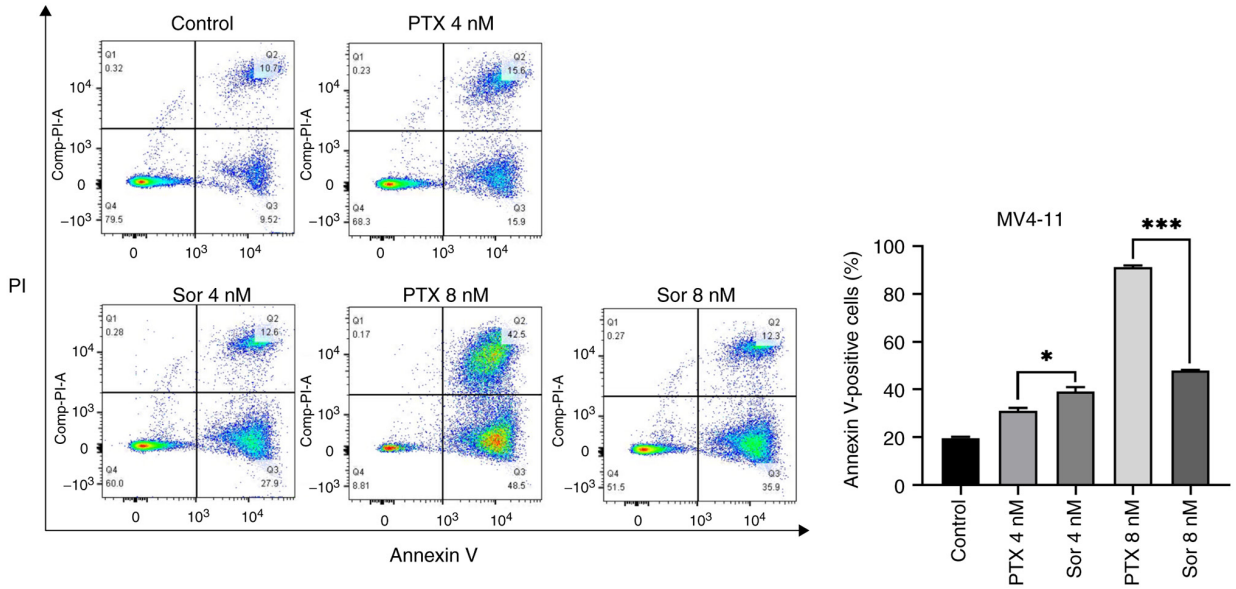

Figure 3. Comparison of PTX and Sor on apoptosis of MV4-11 cells. MV4-11 cells were treated with 4 or 8 nM PTX and Sor for 48 h, followed by flow cytometry assay. *P<0.05, ***P<0.001. PTX, paclitaxel; Sor, sorafenib.

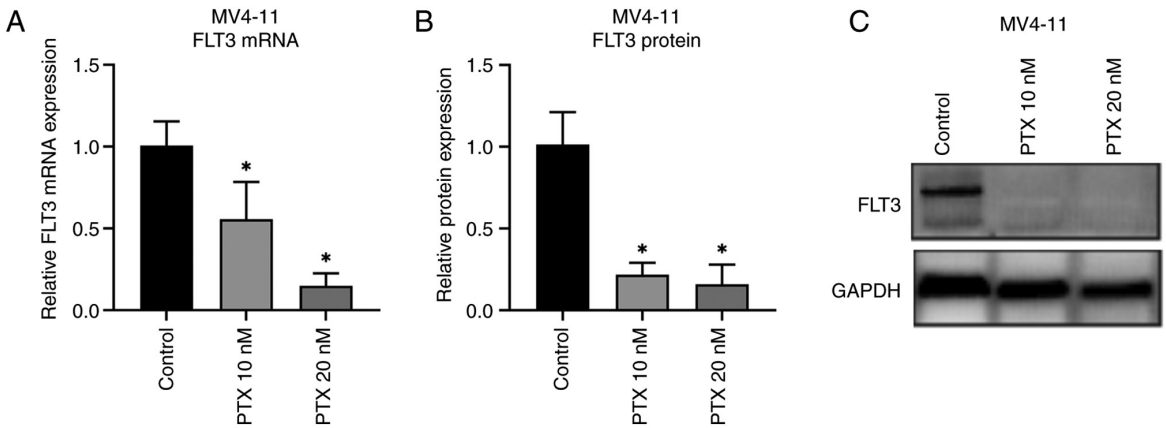

Figure 4. Effect of PTX on expression of FLT3 mRNA and protein. MV4-11 cells were treated with 10 or 20 nM paclitaxel for 48 h, and then the expression of FLT3 mRNA (A) and FLT3 protein (B, C) were detected by PCR and western blotting, respectively. *P<0.05. PTX, paclitaxel.

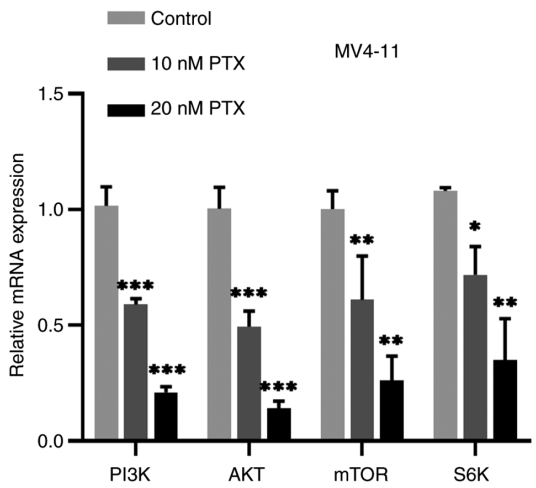

Figure 5. Effect of PTX on mRNA expression of key genes of the PI3K/AKT/mTOR pathway. MV4-11 cells were treated with 10 or 20 nM PTX for 48 h, followed by PCR to detect mRNA expression. *P<0.05, **P<0.01, ***P<0.001. PTX, paclitaxel.

Effect of PTX combined with PKI-587 on cell proliferation and apoptosis of MV4-11 cells. Abnormal activation of PI3K/AKT/mTOR pathway is not only related to the occurrence and development of AML, but also drug resistance (37). As a dual PI3K/AKT/mTOR inhibitor, PKI-587 has been shown to efficiently inhibit cell proliferation, block colony formation and induce apoptosis of sorafenib-resistant AML cells (38). The results of the present study confirmed that PKI-587 also had clear anti-proliferative on MV4-11 cells. As it was observed that PTX could inhibit the PI3K/AKT/mTOR pathway, it was hypothesized that PKI-587 might enhance the effect of PTX in MV4-11 cells, or the two drugs might have synergistic effect. To verify this hypothesis, first, 2 nM PTX combined with 10, 25 or 50 nM PKI-587 was used to treated MV4-11 cells, followed by CCK-8 assay to detect cell viability. As shown in Table II, comparing with PTX or PKI-587 monotherapy, the cell proliferation inhibition rate of the combination group was significantly increased. The synergistic index q value calculated by Kingsdale formula

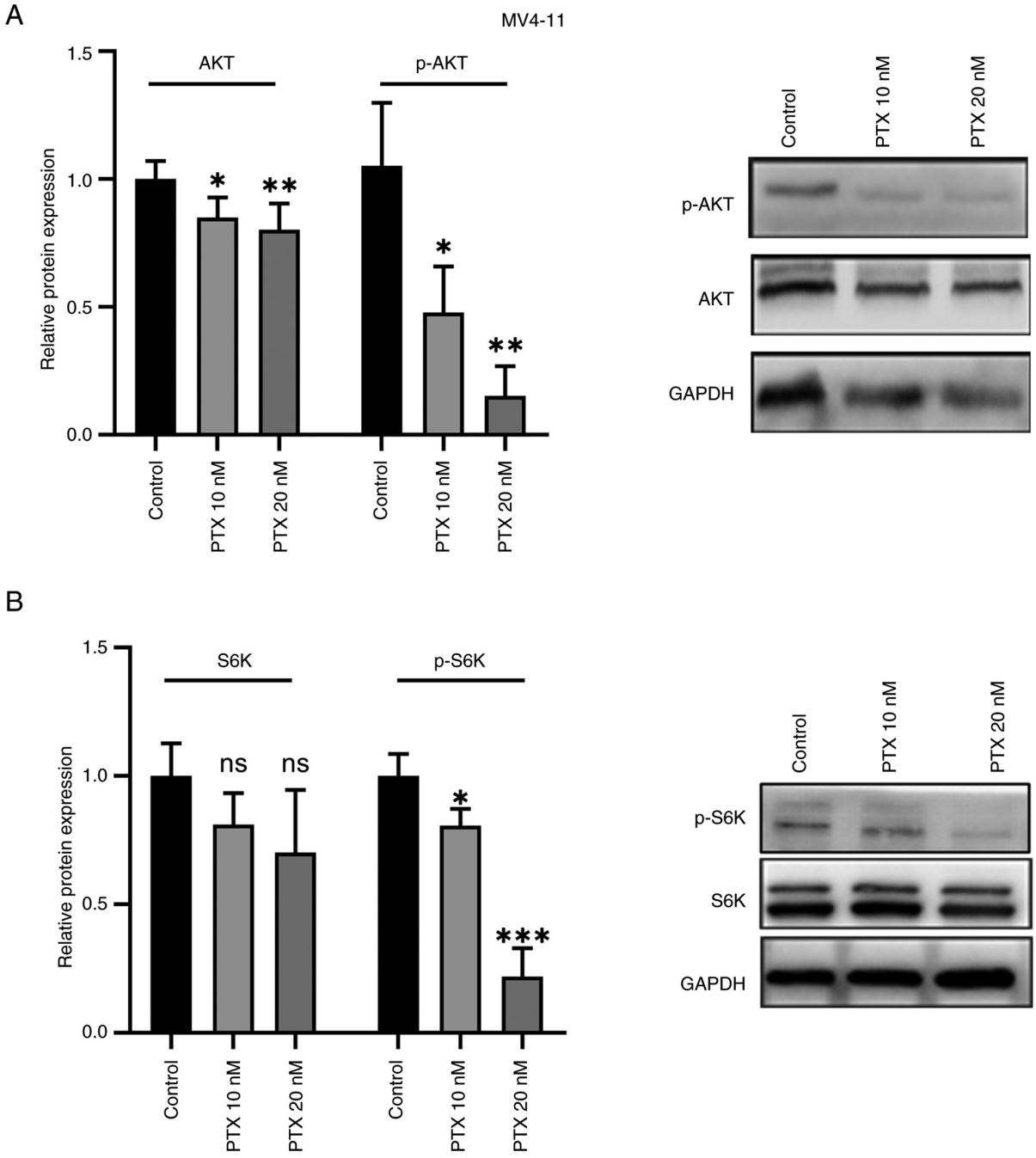

Figure 6. Effect of PTX on protein expression of key genes of the PI3K/AKT/mTOR pathway. MV4-11 cells were treated with 10 or 20 nM PTX for 48 h, and then the expression of (A) AKT and p-AKT and (B) SK6 and p-SK6 protein were detected by western blotting. *P<0.05, **P<0.01, ***P<0.001, ^{ns}P>0.05. PTX, paclitaxel; p-, phosphorylated.

suggested that the combination of PTX and PKI-587 had synergistic or additive effect.

Second, its effect on cells apoptosis was confirmed. From Table II, it can be seen that the synergistic index q value was the largest at 2 nM PTX combined with 10 nM. Therefore, 2 nM PTX combined with 10 nM PKI-587 was selected to treat cells. The results showed that the apoptosis rate of PTX or PKI-587 alone was 27.69±7.45% and 26.42±5.24%, respectively, but at the combination group, the apoptosis rate was up to 76.23±2.55% (Fig. 8). Taken together, it was concluded that PKI-587 might enhance effect of PTX on MV4-11 cells, the two drugs having a synergistic or additive effect. It was also further proved that the proliferation inhibition and pro-apoptosis effect of PTX on MV4-11 cells were related to the PI3K/AKT/mTOR signaling pathway.

Discussion

The prognosis of AML patients carrying the FLT3-ITD mutation remains poor and its 5-year survival rate is only ~20%, despite advances in treatments (39). Thus, there is a pressing need to identify new and effective treatment strategies for these patients (39). PTX is considered an effective antitumor treatment for a variety of malignancies. The present study found

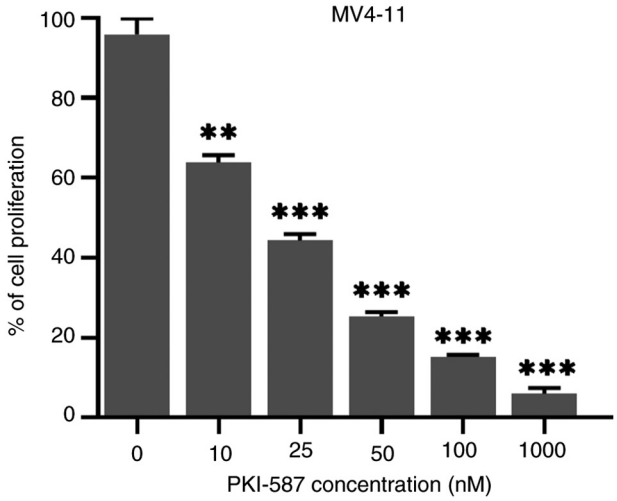

Figure 7. Effect of the pathway inhibitor PKI-587 on cell viability. MV4-11 cells were treated with increasing concentrations of PKI-587 for 48 h, followed by CCK8 assay. **P<0.01, ***P<0.001.

that PTX had a time- and dose-dependent anti-proliferative and apoptosis-inducing effect on MV4-11 cells carrying the FLT3-ITD mutation. PTX also inhibited expression of FLT3 mRNA and protein and exerted a regulatory effect on the PI3K/AKT/mTOR pathway.

Leukemia is a common malignant tumor derived from the hematopoietic system during which bone marrow, peripheral blood and other organs are filled with uncontrollably proliferating primitive white blood cells (40,41). Previous studies have demonstrated anti-proliferative effects of PTX on the leukemia cells, HL-60 (30) and K562 (31,32) and a similar inhibitory effect on MV4-11 cells was predicted. The results showed that PTX had a significant anti-proliferative and apoptosis-inducing effect on MV4-11 cells. To observe and compare its effect on the FLT3-ITD negative leukemia cell lines, THP-1 and K562, both cell lines were treated under the same conditions. PTX could also inhibit cell proliferation and induce apoptosis of THP-1 or K562 cells; however, compared with MV4-11 cells, its effect was weaker. Therefore, it was hypothesized that the anti-proliferative and pro-apoptosis effect of PTX might depend on the leukemia cell type and that it had a more potent effect on FLT3-ITD$^+$ AML cells.

As the FLT3-ITD mutation often indicates high risk in AML patients, several FLT3 inhibitors have been developed and used in clinics for AML patients, such as sorafenib, midostaurin and gilteritinib (42,43). The most widely used is sorafenib, which has been used to treat relapsed or refractory AML patients, or as an adjunct to transplantation, although it is merely a multi-kinase inhibitor (44). To further identify the effect of PTX on MV4-11 cells, its inhibitory and pro-apoptotic effect were compared with sorafenib. The results showed that the IC_{50} values of sorafenib were greater than PTX, indicating the anti-proliferative effect of PTX was stronger than sorafenib. Moreover, the apoptosis rate of sorafenib was lower compared with PTX. However, sorafenib is a multi-kinase inhibitor; in order to improve our understanding of the anti-leukemia effect of PTX, it is necessary to further compare the efficacy of PTX with gilteritinib, a new FLT3/AXL dual inhibitor which has a relatively definite efficacy in relapsed or refractory AML patients (45). Taken together, it was suggested that PTX had distinct effect on MV4-11 cells.

FLT3 is often overexpressed in hematopoietic tumors (6), affecting the development, growth and differentiation of blood cells and promoting leukemia progression. The FLT3-ITD mutation can decrease treatment efficacy and result in adverse outcomes (46) by activating downstream signaling pathways (11,12), such as the PI3K/AKT signaling pathway, which has a great influence on survival, proliferation and differentiation of hematopoietic cells (36). Abnormal activation of the PI3K/AKT signaling pathway associated with reduced overall survival, has been identified in ~60% of AML patients (47,48). A number of studies have shown that mutations or activation of receptor tyrosine kinases, for example, the FLT3-ITD mutation, can stimulate the PI3K/AKT signaling pathway in most human malignancies, including AML (13,49,50). PTX is a potent anticancer drug and several studies have shown that it can inhibit cell proliferation and induce apoptosis by regulating the PI3K/AKT pathway in various solid tumors, such as nasopharyngeal carcinoma (26,51), cervical cancer (27) and lung cancer. Furthermore, the pathway inhibitors or other drugs inactivating the PI3K/AKT pathway can enhance the chemosensitivity of PTX in various cancer cells (52-54). The present study observed that PTX could downregulate the expression of FLT3 mRNA and protein in MV4-11 cells. In addition, upon PTX treatment, the mRNA expression level of PI3K, AKT, mTOR and S6K and the proteins of p-AKT, p-S6K all declined distinctly. However, the changes of AKT, S6K protein were not clear and it was hypothesized that PTX might mainly influence the phosphorylation of AKT and S6K, perhaps through inactivating the PI3K/AKT/mTOR pathway, to affect cell proliferation and apoptosis. It has been reported that PI3K/mTOR pathway inhibitors can inhibit cell proliferation and induce apoptosis of MV4-11 cells and another FLT3-ITD$^+$ AML cell line, MOLM-13 cells. The present study also demonstrated that the resistance of AML cells to sorafenib might have resulted from overexpression of p-AKT and p-S6K proteins of the PI3K/mTOR pathway. Inhibition of this pathway enhanced the anti-leukemia effect of sorafenib, lengthening the life of mice with transplanted tumors (38). The present study found that PKI-587, a dual PI3K/mTOR inhibitor did have cytotoxic effect on MV4-11 cells, which was consistent with a previous study (38). It was also combined with PTX to treat the cells and it was found that this combination showed a significant synergistic or additive effect in inhibiting cell proliferation and inducing apoptosis. These findings further demonstrated that the effect of PTX on MV4-11 cells might be associated with the PI3K/AKT/mTOR pathway. Future studies will further detect whether the effect of PTX increases when the expression of p-AKT and p-S6K are downregulated. However, there is an important question; how PTX regulates the FLT3 is unclear. On the basis of previous studies, it is hypothesized that PTX might inhibit heat shock protein 90 (Hsp90) to downregulate the expression of FLT3.

FLT3 as an important therapeutic target and its expression can be inhibited by a variety of small molecule inhibitors, such as Hsp90, proteasome, RET and other inhibitors (55). Hsp90 is a molecular chaperone that is overexpressed in leukemia cells and its overexpression is associated with indefinite cells proliferation and drug resistance, mainly through forming

Table II. Effect of the combination of paclitaxel and PKI-587 on MV4-11 cell proliferation.

Group	Inhibition rate (%)	q-value	Interaction
Control	3.11±0.63		
2 nM PTX	10.5±3.3		
10 nM PKI-587	14.63±5.93		
25 nM PKI-587	66.68±3.44		
50 nM PKI-587	69.65±5.63		
2 nM PTX + 10 nM PKI-587	35.41±2.63[a]	1.50	Synergy
2 nM PTX + 25 nM PKI-587	83.09±0.56[a]	1.18	Synergy
2 nM PTX + 50 nM PKI-587	82.07±5.87[a]	1.13	Additive

MV4-11 cells were treated with 2 nM paclitaxel alone or in combination with 10, 25 or 50 nM PKI-587 for 48 h followed by CCK8 assay. The Kingsdale formula was used to determine the existence of synergism. Values of q>1.15, 0.85≤ q ≤1.15 and q<0.85 are considered to indicate synergistic, additive and antagonistic effects, respectively. Data is presented as mean ± standard deviation (n=3). [a]P<0.05 vs. paclitaxel monotherapy group.

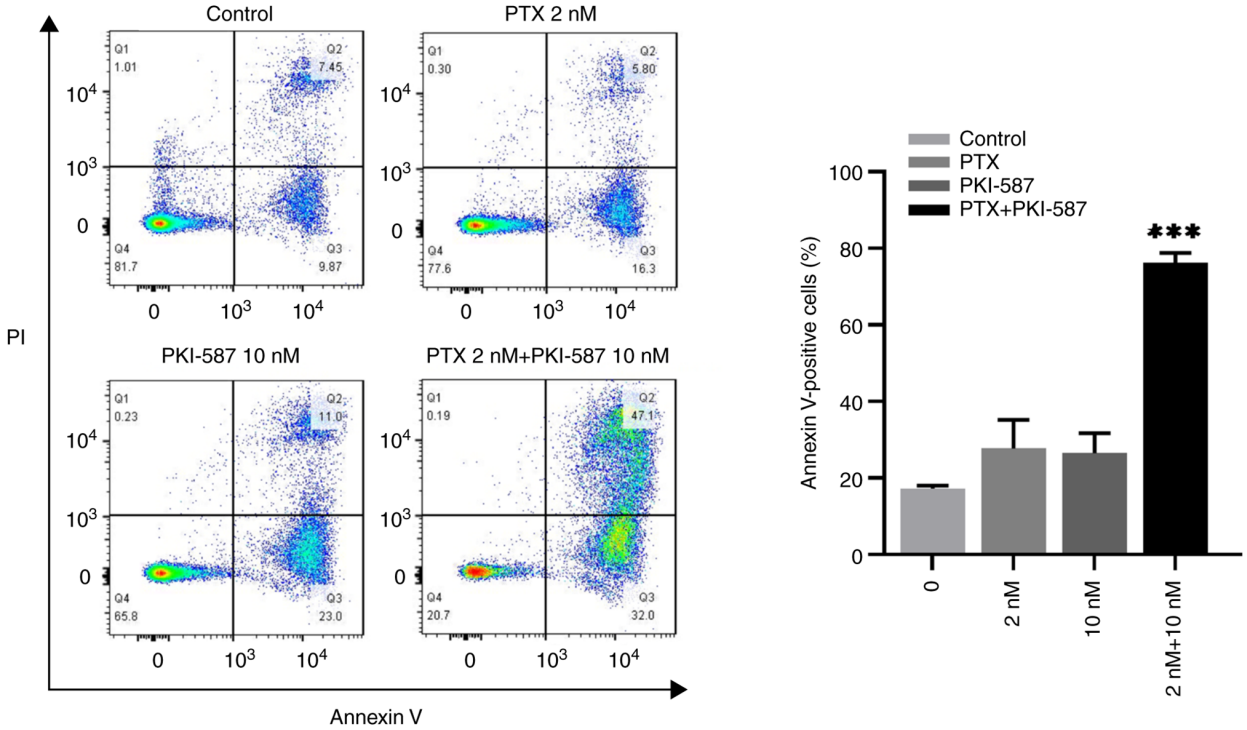

Figure 8. Effect of the combination of PTX and PKI-587 on apoptosis of MV4-11 cells. MV4-11 cells were treated with 2 nM paclitaxel alone or in combination with 10 nM PKI-587 for 48 h, followed by flow cytometry assay. ***P<0.001. PTX, paclitaxel.

multiprotein complexes to stabilize its client proteins (55,56). It was hypothesized that PTX might, through inhibition of Hsp90, downregulate the expression of FLT3 for the following reasons. First, several studies have confirmed that FLT3 is one of the Hsp90 client proteins; Hsp90 inhibitors, such as 17-AAG, can reduce its expression and inhibit proliferation of leukemia cells and induce apoptosis (57-59). Second, AKT is also an Hsp90 client protein; Hsp90 inhibitors can inactivate AKT and downregulate the expression of p-AKT. The present study verified that PTX could reduce the expression of both FLT3 and p-AKT. Third, triptolide and tripterine, isolated from *Tripeterygium wilfordii* Hook f, both are terpenoid compounds. Previous studies have demonstrated that tripterine demonstrates anti-proliferation and pro-apoptosis effect on various leukemia cells by inhibiting the expression of Hsp90 (60-62). It has been reported that triptolide can also inhibit the expression of Hsp90 (63). Considering that PTX is also a terpenoid compound, such as triptolide and tripterine, it was hypothesized that it might also inhibit the expression of Hsp90. For these reasons, it was further hypothesized that PTX might inhibit the expression of FLT3 and p-AKT by reducing the expression of Hsp90. This remains a hypothesis and further work will be conducted to identify its veracity.

In conclusion, the present study revealed the anti-proliferative and apoptosis-inducing effect of PTX on MV4-11 cells and its molecular mechanism, which was connected to

the inhibition of PI3K/AKT/mTOR pathway. The aim of the present study was to establish the potential utility of PTX as a therapy for FLT3-ITD$^+$ AML patients, supported by a theoretical basis. However, the present study was limited by its *in vitro* nature and did not establish the efficacy of PTX in patients. In the future, we will further explore the effects of PTX on primary cells and further animal experiments will be planned which may pave the way for clinical trials.

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Availability of data and materials

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

Authors' contributions

YS and WZ conceived and designed the study. YS, BZ and ZB performed the experiment. RP was responsible for cultivating cells. MW and ZL provided technical guidance. YS wrote the manuscript. WZ revised and finalized the manuscript. YS and WZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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