

Rapamycin enhanced the antitumor effects of doxorubicin in myelogenous leukemia K562 cells by downregulating the mTOR/p70S6K pathway

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Abstract. Chronic myelogenous leukemia (CML) is a common hematological malignancy. Some patients progressing to the blast phase develop chemotherapeutic drug resistance. In the authors' previous study, it was found that the mammalian target of rapamycin (mTOR) pathway was activated in CML and that rapamycin inhibited the proliferation of K562 cells. Targeting the mTOR pathway may be used in combination with chemotherapeutic drugs to enhance their efficacy and overcome multidrug resistance. The aim of the present study was to investigate the effects of rapamycin and doxorubicin on K562 cell proliferation following the combination treatment, and further focus on confirming whether rapamycin enhanced the antitumor effects of doxorubicin by downregulating the mTOR/ribosomal protein S6 kinase (p70S6K) pathway. It was found that rapamycin and doxorubicin significantly decreased the viability of K562 cells. The apoptotic cells were more frequently detected in rapamycin and doxorubicin treatment groups (25.50±1.25%). Both drugs decreased Bcl-2 and increased Bax expression in K562 cells. Rapamycin and doxorubicin also reduced the phosphorylation levels of mTOR and p70S6K. Meanwhile, p70S6K-targeting small interfering (si)RNA and doxorubicin inhibited cell proliferation and regulated key factors of the cell cycle. In addition, the exposure of cells to p70S6K siRNA and doxorubicin significantly increased cell apoptosis, as compared with single treatment.

These results suggested that rapamycin could enhance the antitumor effects of doxorubicin on K562 cells by downregulating mTOR/p70S6K signaling. Targeting the mTOR/p70S6K pathway may be a new therapeutic approach for leukemia.

Introduction

Myeloid leukemia is a common hematological malignancy that is subdivided into acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML). CML is a myelo-proliferative disorder characterized by the Philadelphia chromosome, a fusion of chromosomes 9 and 22, that gives rise to the Bcr/Abl oncogene (1,2). Bcr/Abl kinase inhibitors, such as imatinib, yield an outstanding clinical response in the chronic phase of CML. However, certain patients that progress to the blast phase with a decreased responsiveness develop resistance to Bcr/Abl kinase inhibitors (3). Doxorubicin, one of the most widely used antitumor drugs in leukemia treatment, exerts its proapoptotic effects through disturbing DNA function and inducing DNA damage. To date, the DA regimen (mainly containing doxorubicin and cytarabine) has been the standard treatment for AML, but is not effective for patients in the blast phase. New effective approaches must be explored to enhance the therapeutic efficacy against leukemia.

It has been established that the activation of the mammalian target of rapamycin (mTOR) pathway is closely associated with cell proliferation and differentiation in several types of human cancer, such as breast, lung, and pancreatic cancer (4-7). In addition, the mTOR pathway has been found to be activated in leukemia cells, especially in cell lines expressing Bcr/Abl (8-11). Previous studies demonstrated that the mTOR pathway was activated in myeloid leukemia, including CML, and mTOR inhibitor (rapamycin, sapanisertib) could arrest cells at the G₀/G₁ phase and increase apoptosis in leukemia cells (12-14). mTOR is a serine/threonine kinase which plays important roles in the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Protein Kinase B (AKT)/mTOR pathway. It was reported that PI3K, induced by Bcr-Abl through a direct association with its regulatory subunit, could activate mTOR upon phosphorylation of AKT (15). A recent study showed that a dual PI3K/mTOR inhibitor effectively inhibited cell proliferation and induced apoptosis by downregulating the

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PI3K/mTOR pathway (16). Further evidence that rapamycin combined with celecoxib could enhance the antitumor effects of single treatment on CML cells was provided (17). Therefore, targeting the mTOR pathway may increase cell sensitivity to chemotherapeutic drugs and serve as a new therapeutic approach for leukemia. However, the precise mechanisms through which rapamycin may have synergistic effects with chemotherapeutic drugs in leukemia remained unclear.

The activation of mTOR (phosphorylated-mTOR) could phosphorylate two major downstream targets, ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Upon its phosphorylation, p70S6K induces protein synthesis and cell survival, and increases the translation of 5'-terminal oligopyrimidine tract mRNAs (18-20). This is important for mitogen-induced cell proliferation and chemotherapeutic drug resistance in cancer cells (21,22). The authors' previous study showed that p70S6K and 4E-BP1 were overexpressed and phosphorylated in CML bone marrow and K562 cells (12). It was therefore speculated that rapamycin could enhance the antitumor effects of chemotherapy on leukemia cells through downregulating mTOR signaling.

In the present study, cell growth and apoptosis in K562 cells was investigated following rapamycin and doxorubicin treatment. In addition, the effects of p70S6K-targeting siRNA and doxorubicin on K562 cells were closely investigated. The present study tried to confirm whether rapamycin enhanced the antitumor effects of doxorubicin by downregulating the mTOR/p70S6K pathway in this preliminary study. Determining the underlying mechanism may help define this new therapeutic target for clinical application in hematologic malignancies.

Materials and methods

Chemicals and reagents. The K562 cell line was purchased from the Beijing Institute for Cancer Research. The primary antibodies used in this study include: Rabbit anti-human mTOR (1:1,000; cat. no. 2983), phosphorylated (phospho/p)-mTOR (1:1,000; cat. no. 5536), p70S6K (1:800, cat. no. 2708) and phospho-p70S6K (1:500; cat. no. 9234) were purchased from Cell Signaling Technology, Inc.; Rabbit anti-human β -actin (1:10,000; cat. no. AC026) monoclonal antibody was bought from ABclonal Biotech Co., Ltd.; rabbit anti-human Bax (1:500; cat. no. sc-23959) and Bcl-xL (1:500; cat. no. sc-8392), and mouse anti-human Bcl-2 (1:500; cat. no. sc-509) antibodies were bought from Santa Cruz Biotechnology, Inc.; and rabbit anti-human cyclin dependent kinase (CDK)4 (1:5,000; cat. no. ab108357), CDK6 (1:20,000; cat. no. ab124821), cyclin B1 (1:10,000; cat. no. ab32053) and cyclin D1 (1:200; cat. no. ab16663) were purchased from Abcam. Goat anti-Mouse IgG (H&L), horseradish peroxidase (HRP) conjugated secondary antibody (1:10,000; cat. no. 074-1806; S0002) and Goat anti-Rabbit IgG (H&L), HRP conjugated secondary antibody (1:5,000; cat. no. 074-1506; S0001) were purchased from Affinity Biosciences.

Cell culture and treatment. K562 cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with penicillin (100 U/ml), streptomycin (100 U/ml) and 10%

fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Doxorubicin and rapamycin were diluted in solvent dimethyl sulfoxide (DMSO; Merck KGaA). K562 cells were treated with various concentrations of rapamycin and/or doxorubicin.

Cell counting kit-8 (CCK-8) assay. Doxorubicin was dissolved in DMSO at a concentration of 1 mM as the primary stock solution and stored at 4°C. It was reported that the distribution volume of doxorubicin varies markedly in different patients or for different cell lines (23-26). Therefore, to investigate its effects on cell proliferation, K562 cells were cultured with various concentrations of doxorubicin (0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 μ M) for 24 h at 37°C. After cells were incubated with the CCK-8 reagent (Dojindo Molecular Technologies, Inc.) for 4 h, the cytotoxic effect of doxorubicin on K562 cells was measured on a spectrophotometer microplate reader (BioTek Instruments, Inc.) at a wavelength of 450 nm. Cell viability was assayed and compared to the control group. The sensitivity of cells to doxorubicin was measured by IC₅₀. The concentration of rapamycin (10, 20 and 40 nM) was added to K562 cells according to the authors' previous studies (12,14). The interaction between rapamycin and doxorubicin was assessed using the combination index (CI) (27,28). The CI was used to identify antagonistic (CI>1), additive (CI=1) or synergistic (CI<1) interactions.

Flow cytometry (FCM). K562 cells from different groups treated with rapamycin (20 nM), doxorubicin (0.5 μ M) and a combination of both, were collected and washed twice with phosphate-buffered saline (PBS) and harvested in the buffer (PBS-0.05% trypsin). K562 cells were incubated with 5 μ l Annexin V and 10 μ l propidium iodide (PI) in the dark for 5 min, and then resuspended in 1X binding buffer. Briefly, cell apoptosis was analyzed using an Annexin V/PI apoptosis detection kit [Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.] according to the manufacturer's protocol.

siRNA transfection. K562 cells (1.0x10⁵/ml) were seeded in 6-well plates and transfected with 50 nM siRNA duplexes using Lipofectamine™ 2000 reagent according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). A total of 48 h following transfection of p70S6K siRNA (5'-GACAAAUC CUCAA AUGUA-3') and negative control siRNA (5'-GGC TACGTCCAGGAGCGCA-3'; GE Healthcare Dharmacon, Inc.), the inhibition efficiency at mRNA and protein levels was estimated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis.

Western blot analysis. Total cell proteins were extracted from K562 cells treated with rapamycin and/or doxorubicin using lysis buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl) supplemented with phosphatase inhibitor cocktail. After protein concentration was measured by Gen5 1.0 software (BioTek Instruments, Inc.) on a spectrophotometer microplate reader (BioTek Instruments, Inc.), 20-40 μ g protein was run on a 10-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene difluoride membrane. Following blocking non-specific binding sites with 5% nonfat dry milk in tris-buffered saline with 0.05% Tween at 37°C for 60 min, the membranes were incubated

Table I. Primers for reverse transcription-quantitative PCR amplification.

Primers	Sequence	Product length (bp)
CDK4-sense	5'-AGTTCGTGAGGTGGCTTTAC-3'	
CDK4-antisense	5'-GCCTTGTCAGATATGTCCTTAG-3'	162
CDK6-sense	5'-CTGCCTTGTTGGCAAAGTATC-3'	
CDK6-antisense	5'-CCAGGTAGAAGGACTGCATTAG-3'	229
Cyclin D1-sense	5'-CCACTCCTACGATACGCTACTA-3'	
Cyclin D1-antisense	5'-GGACTGAAAGTGCTTGGAATG-3'	219
Cyclin B-sense	5'-GGTGTCACTGCCATGTTTATTG-3'	
Cyclin B-antisense	5'-CGAAGGAAGTGCAAAGGTAGA-3'	179
p70S6K-sense	5'-CAAGGTGAGGGAGATAGGGATA-3'	
p70S6K-antisense	5'-AAGGAAGGTAGACAGCAGAAAC-3'	134

CDK, cyclin dependent kinase; p70S6K, ribosomal protein S6 kinase.

with primary antibodies against mTOR, p-mTOR, p70S6K, p-p70S6K, Bcl-2, Bax, Bcl-xL, CDK4, CDK6, Cyclin B1 and CyclinD1at 4°C overnight. Next, the membranes were probed with horseradish peroxidase-conjugated secondary antibody (1:5,000) for 60 min at 37°C. Subsequently, the immunoreactive membranes were developed using Pierce™ ECL Western Blotting Substrate (cat. no. 32109; Thermo Fisher Scientific, Inc. cat. no.32109) on Luminescent Image Analyzer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Band density was quantified and normalized to β -actinby Image J software (version 2; National Institutes of Health).

RT-qPCR. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. To obtain cDNA, RT was done with the following conditions: 25°C for 5 min, 42°C for 60 min and 70°C for 15 min. After cDNA was obtained by RT (Promega Corporation), PCR amplification was performed with forward and reverse primers using the SYBR PrimeScript RT-PCR kit (Takara Bio, Inc.) in an Mx3005p instrument (Agilent Technologies GmbH). Amplification was performed with the following thermocycling conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The primers used in the present study are listed in Table I. Cq values of the test genes were normalized to the average Cq values of β -actin. Fold-change was calculated using the $2^{-\Delta\Delta Cq}$ method (29).

Statistical analysis. All experiments were performed at least three times. Values were presented as the mean \pm standard deviation. Statistical differences were performed by one-way analysis of variance with least significant difference Duncan's post-hoc test using SPSS 21.0 for Windows (IBM, Corps.). $P < 0.05$ was considered to indicate a statistically significant difference between groups.

Results

Rapamycin and doxorubicin decreases the viability of K562 cells. The cytotoxic effect of doxorubicin on K562 cells was measured by the CCK-8 assay after treating cells with

increasing concentrations for 24, 48 and 72 h (Fig. 1). As shown in Fig. 1A, when the cells were treated with doxorubicin for 24 h, the cell viability was significantly decreased in a dose-dependent manner ($0.03125-4 \mu\text{M}$; $r = -0.946$; $P < 0.05$). The IC_{50} of doxorubicin treated for 24 h was $3.47 \pm 0.57 \mu\text{M}$. In addition, doxorubicin treatment for 48 and 72 h also significantly inhibited cell proliferation (48h: $r = -0.958$, $P < 0.05$; Fig. 1B; 72 h: $r = -0.968$; $P < 0.05$; Fig. 1C).

In the authors' previously published paper (12), K562 cells were incubated with various concentrations of rapamycin ($5-1,000 \text{ nM}$) and the IC_{50} of rapamycin treatment for 24 h was $174.94 \pm 14.59 \text{ nM}$. To further understand the possible synergistic mechanism of rapamycin and doxorubicin, the cells were incubated with 0.125, 0.25, 0.5 and $1.0 \mu\text{M}$ doxorubicin, combined with 10, 20 and 40 nM rapamycin. The cell survival rates of K562 cells were significantly decreased in the two-drug treatment group compared with the matched single-drug group and the control group ($P < 0.05$; Fig. 1D). Of note, the cell viability in the $0.25 \mu\text{M}$ doxorubicin and 10 nM rapamycin-treated group was 74.4%, and the cell inhibition rate was $\sim 25\%$. In addition, the CI of rapamycin and doxorubicin was analyzed using the Chou-Talalay method (30). As shown in Fig. 1D and E, when the rapamycin concentration was $>10 \text{ nM}$ and the doxorubicin concentration was $>0.2 \mu\text{M}$, the fraction affected (at growth inhibition rates of 25%) is higher than 0.25 and the CI was <1 . According to the definition of the CI (27,28), when CI is <1 , the combination of rapamycin and doxorubicin had a synergistic inhibitory effect on the growth of K562 cells. Among these groups, the cell survival rate of K562 cells treated with 20 nM rapamycin and $0.5 \mu\text{M}$ doxorubicin was $53.4 \pm 8.20\%$. Therefore, 20 nM rapamycin and $0.5 \mu\text{M}$ doxorubicin was chosen for the following experiments.

Rapamycin could enhance the apoptotic effect of doxorubicin on K562 cells. To assess whether apoptosis contributes to cell growth inhibition in rapamycin and doxorubicin treatment groups, the effects of two drugs on K562 cell apoptosis were studied. The apoptotic rates following treatment with rapamycin, doxorubicin and a combination of both were

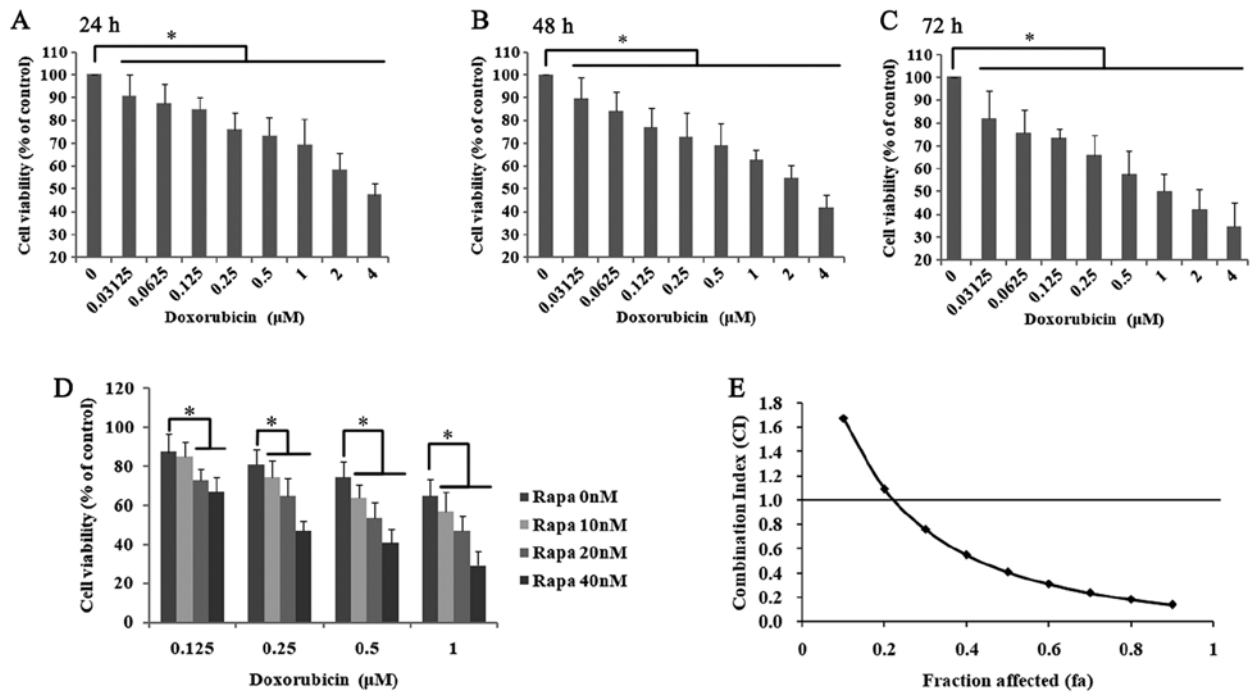


Figure 1. Rapa combined with doxorubicin decreases cell proliferation in K562 cells. Doxorubicin treatment with various concentrations from 0.03125–4 μM for (A) 24, (B) 48 and (C) 72 h. The percentage of cells viability was determined by Cell Counting Kit-8 assay. Data were presented as the mean \pm SD of triplicates. * P <0.05 vs. the solvent control group. (D) Rapa combined with doxorubicin decreased the cell viability of K562 cells. K562 cells were treated with doxorubicin (0.125, 0.25, 0.5 and 1 μM) and Rapa (10, 20 and 40 nM) for 24 h. Control value was taken as 100%, and * P <0.05 vs. the matched single-drug group. Data were presented as the mean \pm SD of triplicates. (E) The CI values were analyzed using the Chou-Talalay method, and the fraction affected (a growth inhibition rates of 25%) is higher than 0.25, $\text{CI}<1$ indicates a synergistic effect. It suggested that Rapa enhanced the anti-proliferative effects of doxorubicin in K562 cells. Rapa, rapamycin; SD, standard deviation.

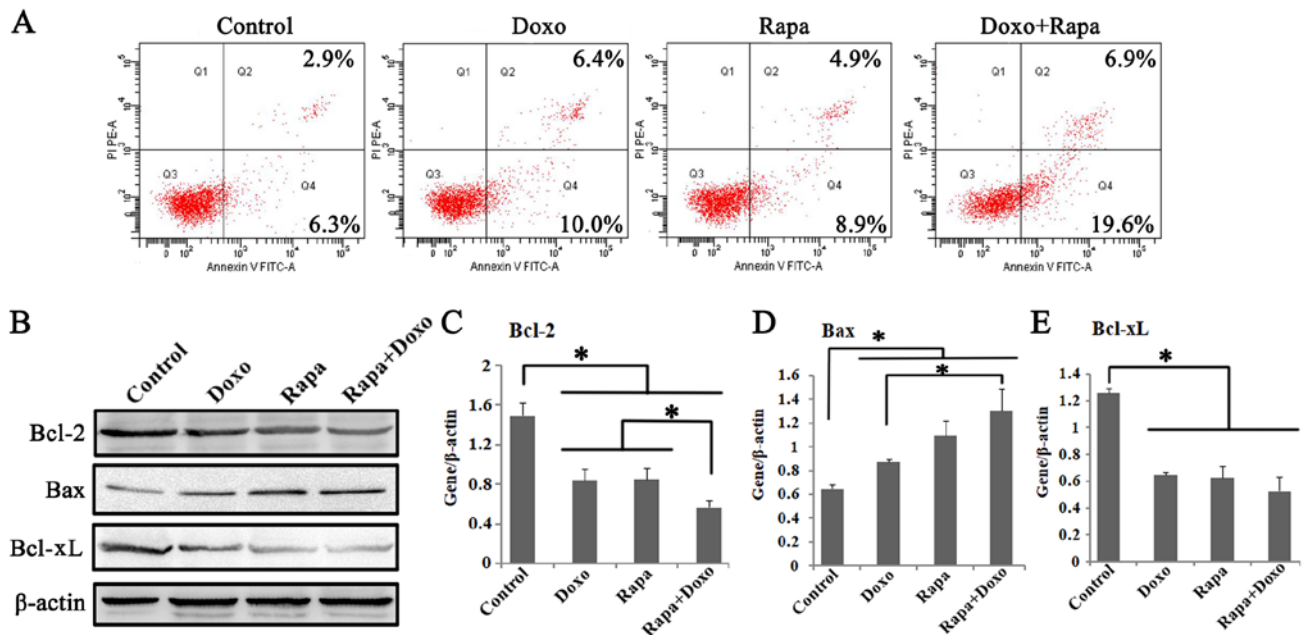


Figure 2. Rapa and Doxo induced cell apoptosis of K562 cells. Cells were incubated with Doxo (0.5 μM) and Rapa (20 nM) for 24 h. (A) Apoptosis of K562 cells was determined by Annexin V/PI apoptosis detection kit. Rapa and Doxo combination significantly increased apoptotic cells, compared with the matched single-drug group. (B) Western blotting showed the expressions of Bcl-2, Bax and Bcl-xL in Rapa, Doxo and the combination treatment group. The protein expression of (C) Bcl-2, (D) Bax and (E) Bcl-xL in K562 cells. Rapa and Doxo significantly decreased expression of (C) Bcl-2 and (E) Bcl-xL, and increase the expression of (D) Bax, respectively. * P <0.05. Data were presented as the mean \pm standard deviation of triplicates. Rapa, rapamycin; Doxo, doxorubicin; FITC, fluorescein isothiocyanate; PI, propidium iodide.

significantly increased compared with in the solvent control group (P <0.05; Fig. 2A). The apoptotic cells, in particular,

were more frequently detected in the rapamycin + doxorubicin treatment group ($25.50 \pm 1.25\%$) compared with rapamycin

Table II. Effect of rapamycin and doxorubicin on apoptosis in K562 cells.

Group	Apoptosis (%)		
	Early	Later	Total
Control	5.63±0.61	2.83±0.50	8.47±0.95
Doxo	9.13±0.90 ^a	5.73±0.65 ^a	14.87±1.34 ^a
Rapa	8.30±0.66 ^a	3.93±0.87	12.23±1.37 ^a
Doxo+Rapa	18.10±1.37 ^{a,b}	7.40±0.62 ^{a,b}	25.50±1.25 ^{a,b}

^aP<0.05 vs. the solvent control group; ^bP<0.05 vs. the single drug treatment. Doxo, doxorubicin; Rapa, rapamycin.

(12.23±1.37%) and doxorubicin (14.87±1.34%) treatment groups (P<0.05; Fig. 2A; Table II).

To further investigate the apoptotic pathway activated by rapamycin and doxorubicin, the effects of both drugs on the expression of proteins that are pivotal for apoptosis, including Bcl-2, Bax, and Bcl-xL, were studied. As shown in Fig. 2B, the significant downregulation of Bcl-2 and Bcl-xL and significant upregulation of Bax were observed in rapamycin, doxorubicin, and the combination treatment group (P<0.05; Fig. 2C-E). Of note, compared with the single-treatment group, a significantly decreased Bcl-2 and increased Bax expression were observed in the combination treatment cells (P<0.05). It indicated that a decrease in the Bcl-2/Bax ratio might be involved in the apoptotic pathways induced by rapamycin and doxorubicin in K562 cells. The above findings confirmed that rapamycin could enhance the apoptotic effect of doxorubicin on K562 cells.

Inactivation of the mTOR/p70S6K pathway in rapamycin- and doxorubicin-treated K562 cells. The effects of rapamycin and doxorubicin on the mTOR/p70s6k pathway in K562 cells were investigated by western blot analysis. As shown in Fig. 3A, although the total mTOR expression showed slight changes in K562 cells, the phosphorylation level of mTOR (Ser-2448) treated with rapamycin, doxorubicin and a combination of both was significantly decreased compared with in the solvent-treated control group (P<0.05; Fig. 3A). The expression of p70S6K was also investigated. Rapamycin, doxorubicin and a combination of both induced a significant decrease in the total and the phosphorylation level of p70S6K (Thr-389) in K562 cells (P<0.05; Fig. 3B). In addition, p70S6K and its phosphorylated form were significantly decreased in cells treated with both drugs compared with those treated with rapamycin or doxorubicin alone (P<0.05; Fig. 3B).

p70S6K siRNA decreases the expression of p70S6K in K562 cells. To understand the role of p70S6K in the rapamycin pathway, K562 cells were transfected with targeting siRNA to block p70S6K. The successful knockdown of p70S6K was confirmed by RT-qPCR and western blot analysis. Compared with the control siRNA group, the mRNA expression of p70S6K was significantly decreased to ~1/5 in p70S6K targeting siRNA cells (P<0.05; Fig. 4A). Furthermore, western blot analysis

results showed that the p70S6K expression was markedly decreased in the p70S6K siRNA treatment group (Fig. 4B). The present data showed that the p70S6K-targeting siRNA effectively downregulated the expression of p70S6K.

p70S6K downregulation enhances the inhibitory effect of doxorubicin on cell proliferation. To investigate the role of p70S6K in the inhibitory effect of rapamycin and doxorubicin on cell proliferation, cell viability was further detected following treatment with p70S6K siRNA and doxorubicin. The CCK-8 assay results showed that p70S6K-targeting siRNA and doxorubicin treatment significantly decreased cell proliferation compared with the p70S6K siRNA, doxorubicin and control siRNA groups (P<0.05; Fig. 4C).

Certain critical proteins involved in cell proliferation were also investigated by western blot analysis and RT-qPCR. The knockdown of p70S6K and doxorubicin treatment significantly decreased the mRNA and protein expression of CDK4, CDK6, cyclin D1 and cyclin B1 (P<0.05; Fig. 4D and E). These results may suggest that rapamycin could enhance the inhibitory effect of doxorubicin on cell proliferation by downregulating the mTOR/p70S6K pathway in K562 cells.

p70S6K downregulation enhances the doxorubicin-induced K562 cell apoptosis. To study whether the cytotoxic effects of treatment with p70S6K siRNA and doxorubicin are associated with enhanced apoptosis, cell apoptosis was examined by FCM. K562 cells were treated with p70S6K siRNA, doxorubicin alone and a combination of both. The results showed that the knockdown of p70S6K and doxorubicin treatment significantly increased the early, late and total apoptotic rates, as compared with the control group (P<0.05; Fig. 5A and B). Compared with single treatment, the exposure of cells to p70S6K siRNA and doxorubicin significantly increased apoptosis (P<0.05; Fig. 5A and B). In addition, western blot analysis results showed a decreased expression of Bcl-2 and increased expression of Bax in K562 cells treated with p70S6K siRNA and doxorubicin (Fig. 5C). These data suggested that blocking p70S6K signaling could enhance doxorubicin-induced K562 cell apoptosis.

Discussion

Despite the fact that Bcr/Abl kinase inhibitors have significantly improved life expectancy in the majority of patients in the chronic phase of CML, certain patients progressing to the blast phase will develop Bcr/Abl-independent mechanisms of resistance. This patient population requires chemotherapeutics, including doxorubicin and cytarabine, or needs an alternative drug target that can be inhibited with a novel compound. Certain studies have shown that mTOR signaling is frequently activated in CML (8-12). In addition, Bcr/Abl kinase could regulate PI3K activity through its regulatory subunit and mTOR was subsequently activated by the phosphorylation of AKT in CML cell lines (15). It was found in the authors' previous studies that rapamycin markedly inhibited cell growth and enhanced the antitumor effects of celecoxib on CML cells by downregulating the mTOR pathway (12,17). It is well known that mTOR exists in two multiprotein complexes, mTORC1 and mTORC2. The main function of mTORC1

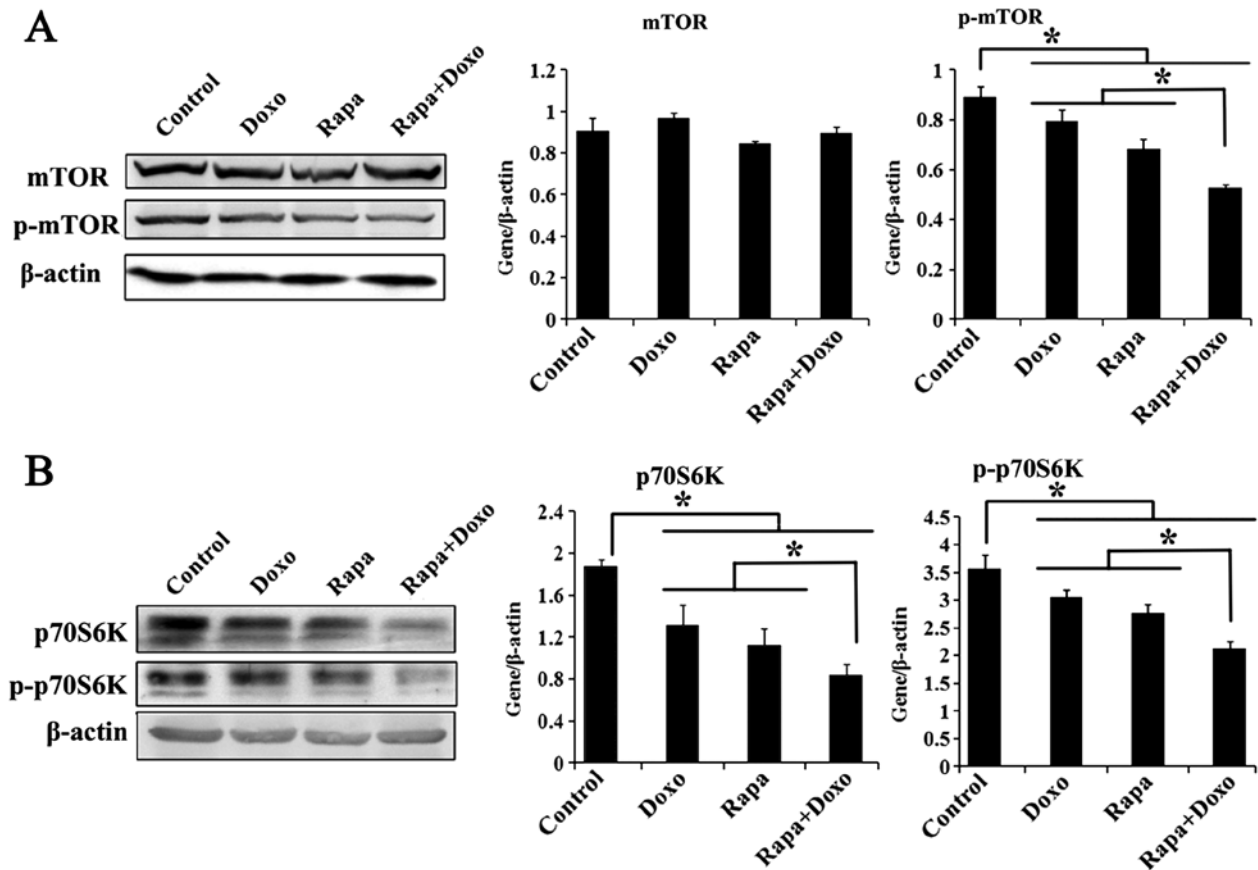


Figure 3. Rapa and Doxo inhibit expression of mTOR/p70S6K signaling. Cells were cultured with Doxo (0.5 μ M), Rapa (20 nM) or combination treatment. Total protein was extracted, followed by immunoblotting with mTOR, p-mTOR (Ser-2448), p70S6K, p-p70S6K (Thr-389) and β -actin antibodies. Experiments were repeated 3 times and data were presented as the mean \pm standard deviation of triplicates. (A) mTOR phosphorylation (Ser-2448) was significantly decreased compared with the solvent control group and the matched single-drug group. *P<0.05. (B) Rapa, Doxo, alone or combination treatment decreased the expression of p70S6K and its phosphorylation (Thr-389) *P<0.05. Rapa, rapamycin; Doxo, doxorubicin; p-mTOR, phosphorylated-mammalian target of rapamycin; p70S6K, ribosomal protein S6 kinase.

which phosphorylates p70S6K and 4E-BP1, is sensitive to nutrients and rapamycin. In contrast to mTORC1, mTORC2 which is insensitive to rapamycin, could phosphorylate AKT at Ser473 and forms a feedback loop (31). Certain studies have demonstrated that an mTOR inhibitor, combined additively or synergistically with several chemotherapies, such as paclitaxel, doxorubicin and carboplatin, could inhibit cancer cells proliferation *in vitro* (32-34). Collectively, targeting the mTOR pathway may be a new therapeutic approach. However, the mechanisms through which rapamycin may have synergistic effects with doxorubicin on leukemia cells remain virtually unknown.

To further explore the possible synergistic mechanisms of rapamycin and doxorubicin on cell growth, K562 cells were cultured with a combination of various concentrations. According to the authors' published paper (12), the IC_{50} of rapamycin treatment for 24 h is 174.94 ± 14.59 nM. It was reported that the distribution volume of doxorubicin varies markedly in different patients or for different cell lines (23-26). Therefore, to investigate effects of doxorubicin on cell proliferation, K562 cells were cultured with various concentrations from 0.03125-4 μ M. The results showed that doxorubicin decreased K562 cells in a dose-dependent manner and The IC_{50} of doxorubicin treatment for 24 h was 3.47 ± 0.57 μ M. The cell survival rates of K562 cells were markedly lower in two-drug treatment group than in the

matched single-drug group. Meanwhile, when the rapamycin concentration was >10 nM, the doxorubicin concentration was >0.2 μ M, and the growth inhibition rates is higher than 25%, CI was less than 1 (CI<1). Co-incubation markedly increased the response of K562 cells to doxorubicin, indicating that rapamycin synergistically enhance the anti-proliferative effects of doxorubicin in K562 cells.

In addition, cell apoptosis was measured in rapamycin- and doxorubicin-treated K562 cells. The results showed that individual treatment can induce cell apoptosis and regulate the expression of Bcl-2, Bax and Bcl-xL. As compared with single-drug treatment, rapamycin combined with doxorubicin increased cell apoptosis. It has been demonstrated that the Bcl-2 family members regulate the cell apoptotic pathway. In particular, increasing Bax expression and/or decreasing Bcl-2 expression may induce apoptosis (35). The present results showed that both drugs induced Bcl-2 downregulation and Bax upregulation, resulting in a decrease in the Bcl-2/Bax ratio. It was also found that rapamycin and doxorubicin can decrease the phosphorylation of mTOR and p70S6K. A recent study found combined treatment with rapamycin and melatonin to be associated with increased apoptosis and mitophagy in head and neck cancers (33). These findings suggested that rapamycin enhances the apoptotic effect of doxorubicin on K562 cells through the mTOR/p70S6K pathway.

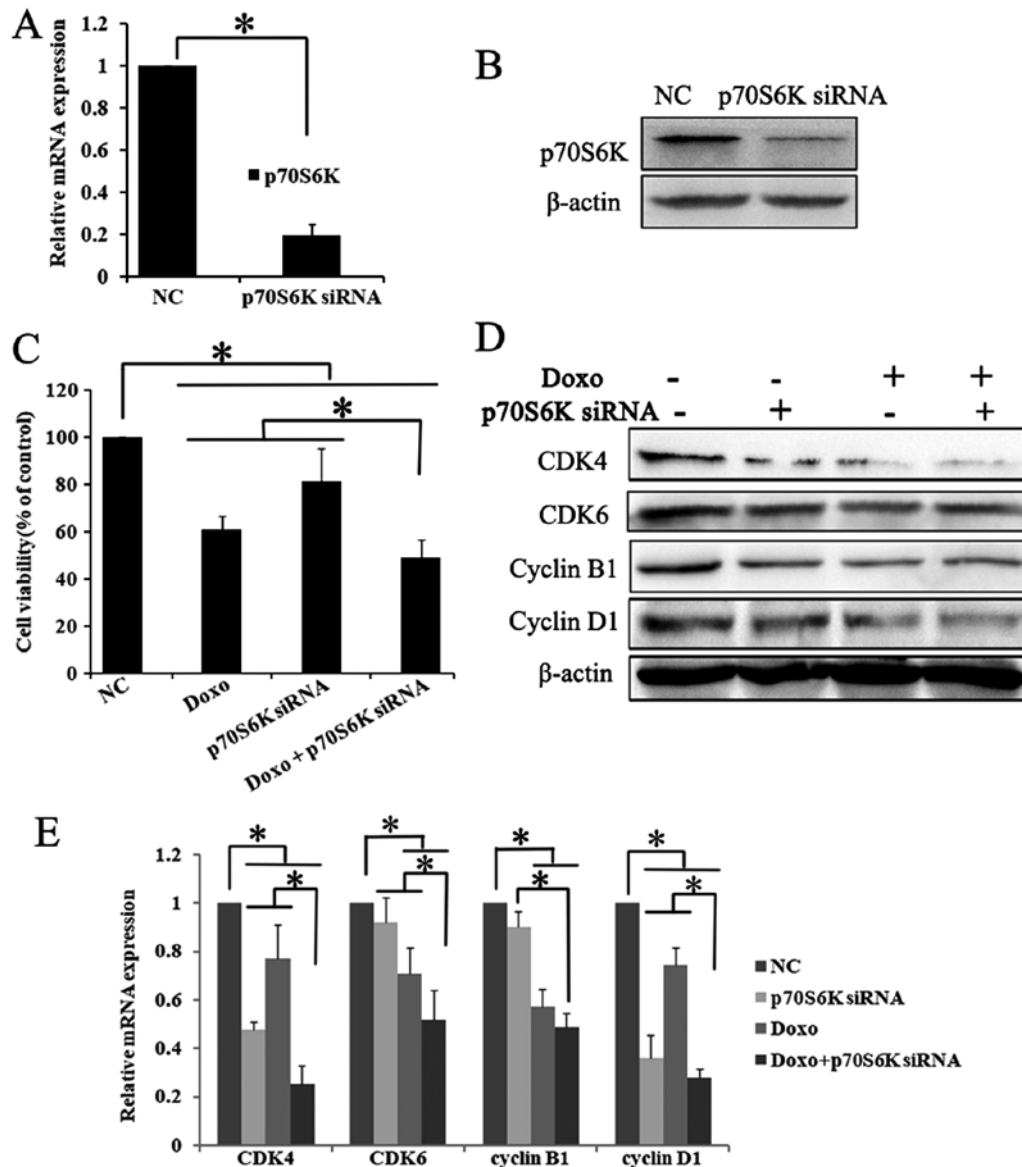


Figure 4. p70S6K knock down enhances the anti-proliferative effects of Doxo in K562 cells. K562 cells were cultured and transfected with p70S6K targeting siRNA for 48 h. Successful knockdown of p70S6K was confirmed by (A) RT-qPCR and (B) western blot assays. The results showed that targeting siRNA significantly decreased the expression of p70S6K, compared with the control siRNA group. * $P < 0.05$. Data were presented as mean \pm SD of triplicates. (C) Cells were cultured with Doxo (0.5 μ M) after p70S6K knockdown, the cell viability was determined by Cell Counting Kit-8. The exposure of p70S6K knockdown and doxorubicin alone or combination significantly inhibited cell viability. * $P < 0.05$. The values are represented as the mean \pm SD. The expression of cell cycle molecules were detected using (D) western blotting and (E) RT-qPCR. The expression of CDK4, CDK6, cyclin B1 and cyclin D1 were significantly decreased in p70S6K knockdown and Doxo treatment group compared with the single treatment group and the control group. * $P < 0.05$. Data were presented as mean \pm SD of triplicates. CDK, cyclin dependent kinases; SD, standard deviation; RT-q, reverse transcription-quantitative; si, small interfering; Doxo, doxorubicin; p70S6K, ribosomal protein S6 kinase.

As is known, 4EBP1 and p70S6K are the two most important downstream proteins of the mTOR pathway. The phosphorylation of 4E-BP1 (Ser37; Thr46; Ser65; Thr70) regulates cap-dependent transcription and translation of a great number of proteins. On the other hand, activated p70S6K could increase the translation of 5'-terminal oligopyrimidine tract mRNAs, regulates protein synthesis and plays a critical role in controlling cell growth (36). Extensive evidence has revealed that the overexpression and phosphorylation of p70S6K promotes cell proliferation, angiogenesis and suppression of apoptosis *in vitro* (37,38), whereas, the p70S6K specific inhibitor (PF-4708671) has inhibitory effects on non-small cell lung cancer *in vitro* and *in vivo* (39). High p70S6K phosphorylation

has also been reported to be an independent biomarker of poor prognosis in right-sided colorectal cancer (20). Similar observations of the p-p70S6K prognostic function have been made for lung, esophageal and ovarian cancer, and hepatocellular carcinoma (40-42).

Studies have previously demonstrated that overexpression of p70S6K in tumor tissues contributes to chemotherapy resistance (43). High levels of p-p70S6K were involved in selumetinib resistance, and the inhibition of p70S6K could reverse this chemotherapeutic resistance in colorectal cancer (44). The authors have previously found that the inhibition of p70S6K by rapamycin was additionally associated with an increased number of G₀/G₁ cells and apoptotic cells in

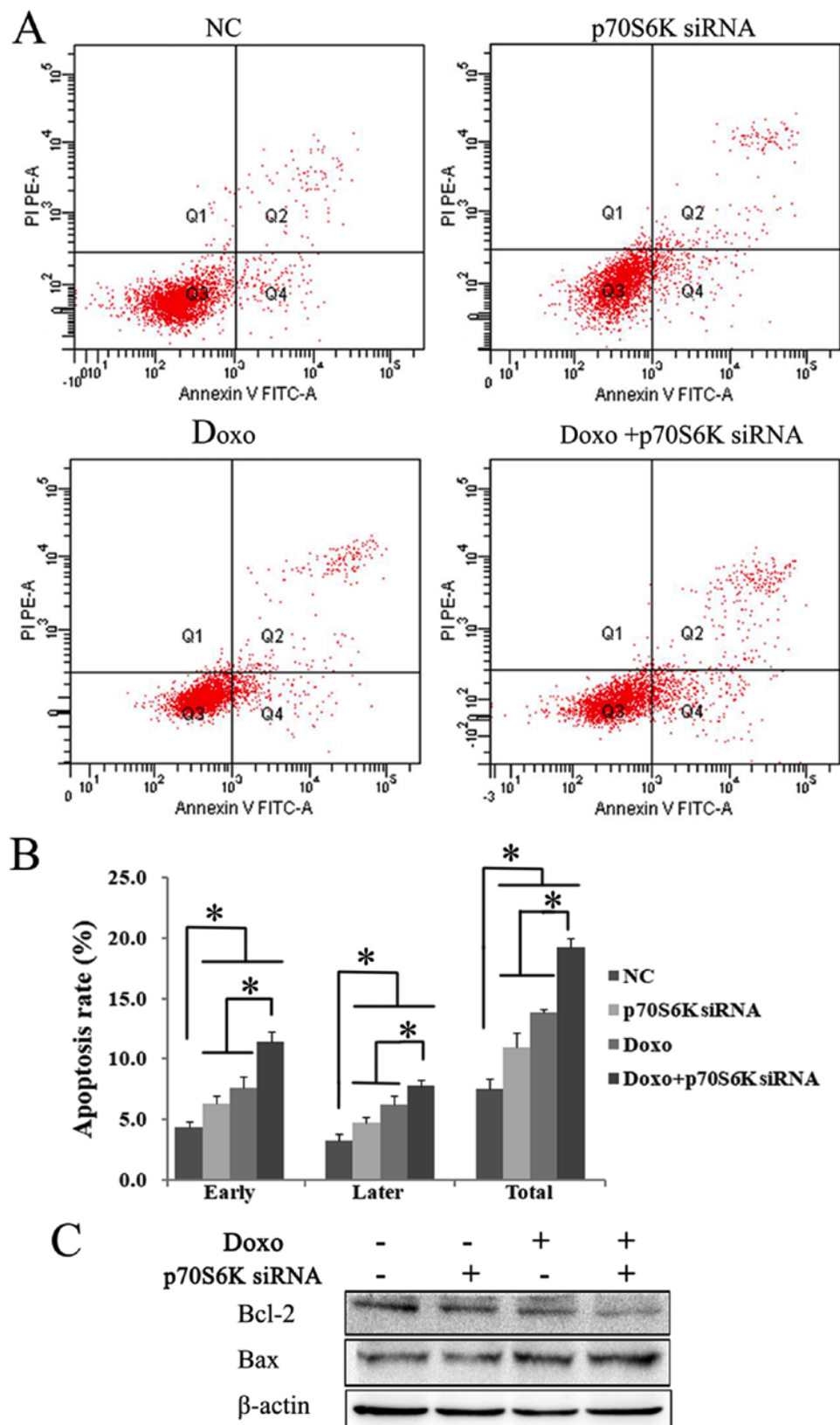


Figure 5. p70S6K knock down enhances Doxo-induced cell apoptosis. (A) Cell apoptosis were detected by flow cytometry analysis. (B) p70S6K knock down and Doxo treatment significantly increased the early apoptotic, late apoptotic and total apoptotic cells than the single treatment group and the control group. * $P < 0.05$. The values are represented as the mean \pm standard deviation. (C) Western blot results showed the significantly lower expression of Bcl-2 and higher expression of Bax in p70S6K knockdown and Doxo treatment group. It suggested that p70S6K knock down enhance Doxo-induced cell apoptosis. Doxo, doxorubicin; NC, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide; p70S6K, ribosomal protein S6 kinase.

leukemia, while rapamycin combined with celecoxib enhanced this effect (12,17). A more recent study showed p70S6K1

suppressed autophagy through inhibiting AMPK and JNK in a TAK1-dependent manner (45).

In the present study, a p70S6K-targeting siRNA was used as an inhibitor to knockdown p70S6K and more attention was paid to investigate its mechanism. It was found that p70S6K knockdown and doxorubicin treatment decreased cell proliferation, compared with the single treatment. The p70S6K siRNA and doxorubicin clearly decreased the expression of CDK4, CDK6, cyclin D1 and cyclin B1, as compared with the control and single-treatment groups. These molecules are key elements in the cell cycle and serve an essential role in the progression of various types of cancer. Among these, cyclin D1 binding to CDK4 is triggered at the early stage of the G₁ phase. The inhibition of cyclin D1 or CDK4 partly restores G₁/S arrest and sensitizes cells to doxorubicin-mediated cell apoptosis, significantly overcoming doxorubicin resistance (46,47). The authors have previously demonstrated that rapamycin arrested K562 cells at the G₀/G₁ phase through mTOR and its downstream molecules (12,15). The present study showed that the downregulation of p70S6K could inhibit CDK4 and cyclin D1 expression. In addition, compared with single treatment, the knockdown of p70S6K and doxorubicin treatment significantly increased cell apoptosis. Furthermore, western blot analysis results showed that p70S6K siRNA and doxorubicin regulate the expression of Bcl-2 and Bax in K562 cells. It suggested that rapamycin synergistically enhanced the doxorubicin-induced inhibition of cell proliferation and apoptosis through the mTOR/p70S6K pathway in K562 cells.

In conclusion, these findings showed that rapamycin and doxorubicin synergistically inhibited cell growth, induced apoptosis, and decreased the expression of mTOR/p70S6K. In addition, p70S6K blocking by siRNA, combined with doxorubicin, could inhibit cell proliferation and induce apoptosis in K562 cells. The authors' preliminary results suggested that rapamycin may enhance the antitumor effects of doxorubicin on K562 cells by downregulating mTOR/p70S6K signaling. Targeting the mTOR pathway may be a more suitable therapeutic approach for leukemia.

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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 and LX designed the study, analyzed the data and wrote the manuscript. WL and QW performed the experiments and prepared the figures. HH designed the study, prepared the figures and analyzed the data. JL and LX performed critical

revision of the manuscript and supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Hebei General Hospital (Shijiazhuang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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