Synergistic effects of LY294002 and ABT199 on the cell cycle in K562, HL60 and KG1a cells

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Abstract. The aim of the present study was to investigate the synergistic effect of LY294002 (a PI3K inhibitor) and ABT199 (a BCL2 inhibitor) on the cell cycle in acute myeloid leukemia (AML). The optimal concentration and duration of combined LY294002 and ABT199 were determined in human erythroleukemia (K562), promyelocytic leukemia (HL60) and myeloid leukemia (KG1a) cell lines. The mRNA and protein expression levels of cell cycle-related molecules, including S-phase kinase-associated protein 2 (Skp2), p27, BCL2, Bax, cleaved caspase 3 (caspase-3) and caspase 9 (caspase-9) were detected via reverse transcription-quantitative PCR and western blot analysis, respectively. At the molecular level, LY294002 and ABT199 combination treatment significantly downregulated Skp2, Bcl2, procaspase-3 and procaspase-9 expression levels, but markedly upregulated p27, Bax, cleaved caspase-3 and caspase-9 expression levels in K562, HL-60 and KG1a cells. The results of the present study demonstrated that LY294002 and ABT199 combination treatment may serve as a novel therapeutic strategy for AML.

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Abbreviations: AML, acute myeloid leukemia; BCL2, B-cell leukemia/lymphoma 2; Bax, BCL2-associated X; caspase-3, caspase 3; caspase-9, caspase 9; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; EIF4EBP1, eukaryotic translation initiation factor 4E binding protein 1; P27, p27 protein; PBS, phosphate buffer; PI3K, phosphoinositide 3-kinase; PI, propyl iodide solutions; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; mTOR, rapamycin kinase; RPS6, ribosomal protein S6; RPS6KB1, ribosomal protein S6 kinase B1; Skp2, S-phase kinase associated protein 2

Key words: acute myeloid leukemia, LY294002, ABT199, cell cycle, therapeutic strategy

Introduction

Acute myeloid leukemia (AML) is common in adults and children (1), but is typically considered a disease of the elderly population (2). AML is characterized by the rapid growth of the myeloid lineage of blood cells and the malignant transformation of hematopoietic stem/progenitor cells (3,4). Malignant precursor cells accumulate in the blood and bone marrow, resulting in acute symptoms, including anemia, infections, bleeding and bruising, bone pain, bone marrow failure and death (5). Elderly patients with AML have a markedly less favorable prognosis due to increased resistance to standard cytotoxic agents (6-8). In addition, patients with AML who respond to chemotherapy often relapse later in life (7,9,10). Yu *et al* (11) reported that relapse typically occurred within the first 3 years from the end of chemotherapy in young patients. Therefore, preventing chemoresistance in a selective manner and identifying a novel therapeutic strategy are important for improving the cure rate of AML.

The PI3K/AKT signaling pathway serves an important role in maintaining cell proliferation and survival, and dysregulation of the signaling pathway is involved in various malignancies, including AML (12-16). Directly stimulating the mitochondrial apoptosis signaling pathway is a novel therapeutic strategy to target cancer cells (17). The BCL2 protein family regulates the mitochondrial apoptosis signaling pathway, and aberrant upregulation of BCL2 is related to carcinogenesis and drug resistance (18). BCL2 overexpression has also been reported in AML (19). Moreover, BCL2 overexpression can increase leukemia fitness, render intrinsic chemoresistance, and contribute to the survival of minimal residual quiescent leukemia stem cells that are responsible for AML relapse (20,21). Activation of the PI3K/AKT/mTOR signaling pathway and BCL2 upregulation are related to stroma-mediated AML survival (22-26).

LY294002, an inhibitor of PI3K, is widely used to study the role of the PI3K/AKT signaling pathway in transformed cells (27,28). In some cancer cell lines, LY294002 can induce apoptosis and increase sensitivity to chemotherapeutic drugs (29-31). It is reported that LY294002 enhances chemosensitivity of K562 cells to Adriamycin (32). ABT199 is a second-generation, specific antagonist of BCL2 (3). At nanomolar concentrations, ABT199 induces apoptosis in various

chemosensitive and chemoresistant AML stem and progenitor cells, and inhibits leukemic progression (3). In addition, a combination of ABT199 hypomethylating agents showed an encouraging response in patients with newly diagnosed AML (33). At present, the most promising drugs for targeted treatment of AML are inhibitors that regulate metabolism or signaling pathways (34). However, it is difficult for single-target inhibitors to produce significant and sustained effects. The scientific and reasonable combination of multi-pathway or multi-target drugs is a research hotspot. Therapeutic strategies targeting the key molecules in the PI3K/AKT and cell apoptosis signaling pathways, such as LY294002 and ABT199, may improve therapeutic efficacy in patients with AML.

The aim of the present study was to investigate whether LY294002 and ABT199 exerted a synergistic effect on AML cell apoptosis and the cell cycle. The result of the present study may provide insight for the combined application of LY294002 and ABT199 in the treatment of AML, thus providing a novel therapeutic strategy for the disease.

Materials and methods

Cell lines and cell culture. Human erythroleukemia (K562) and promyelocytic leukemia (HL60) cell lines were purchased from Wuhan Punosai Life Technology Co., Ltd. The K562 cell line was isolated and established from human leukemia cells, which can grow *in vitro* over a long period of time. Based on the characteristics of a short proliferation cycle, as well as stable growth and metabolism, the K562 cell line is a commonly used model cell line in biomedical research (35). The HL60 cell line is typically used to study how certain blood cells form, providing continuous human cells for the study of molecular events in granulocyte differentiation and the physiological effects of this process, drug action and viral components (36). The human myeloid leukemia cell line (KG1a) was purchased from Shanghai Xinyu Biological Technology Co., Ltd. The KG1a cell line is morphologically similar to AML, displaying significant polymorphisms (37). K562, HL60 and KG1a cells were cultured in medium (IMDM medium for K562 cells; RPMI-1640 medium for HL60 and KG1a cells) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 1% 100 IU/ml penicillin and 100 mg/l streptomycin (Beijing Solarbio Science and Technology Co., Ltd.) at 37˚C in a humidified atmosphere with 5% CO_2.

Reagents. PI3K inhibitor (LY294002) and BCL2 inhibitor (ABT199) were purchased from Biyuntian Technology, Inc. To make a 10 mM stock solution, 25 mg LY294002 was dissolved in 8.13 ml DMSO. Similarly, to make a 10 mM stock solution, 25 mg ABT199 was dissolved in 2.9 ml DMSO. Cell medium was used for the preparation of a concentration gradient of LY294002 and ABT199. The concentration gradient of LY294002 was as follows: 0.5, 0.57, 0.97, 1.5, 2.5 and 5 μM. The concentration gradient of ABT199 was as follows: 3, 8, 15, 20, 30 and 50 nM. The related effects of LY294002 and ABT199 have been previously investigated (38,39). Stable compounds are considered optimal for drug research. It has been hypothesized that LY294002 and ABT199 do not undergo degradation or other alterations in activity in the medium, and their chemical properties are relatively stable.

Cell counting kit-8 (CCK-8) assay. K562, HL60 and KG1a cell suspensions were prepared and seeded (100 μl/well; three replicate wells) into 96-well plates. Cells were cultured for 24 h to allow adherence. Subsequently, the cells were treated with LY294002 and ABT199 for 24, 36, 48 or 72 h. Then, 10 μl CCK-8 solution (Biyuntian Technology, Inc.) was added to each well and cultured for 1 h. Absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Dose-effect relationship of single and combination treatment of drugs on cells. The cell activity value was calculated according to the optical density value obtained via the CCK-8 assay. Based on the cell activity value, the IC_{50} value was determined using an online calculator (www.aatbio.com/tools/ic50-calculator). The smaller the IC_{50} value, the more suitable the treatment was for selection. A synergistic effect was observed when the inhibitory rate of the combination treatment was greater than the sum of the inhibitory rates of the two single drugs, which had reference significance. In the present study, Jin's Formula was used to evaluate the synergistic effect (40). The formula is as follows: Q=Eab/(Ea + Eb-Ea x Eb), where Ea is the inhibition rate of LY294002 treatment, Eb is the inhibition rate of ABT19 treatment and Eab is the inhibition rate of LY294002 and ABT199 treatment. $Q<0.85$ indicates that the combined effect of the two agents is antagonistic, $0.85\leq Q<1.15$ indicates that the combined effect of the two agents is additive and $Q\geq1.15$ indicates that the combined effect of the two agents is synergistic. Based on Jin's Formula, the Q-value in the three cell lines was ≥1.15, which indicated a synergistic effect of LY294002 and ABT199.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from K562, HL60 and KG1a cells using TRIzol® according to the manufacturer's protocol. RNA concentration and purity were determined using a nucleic acid concentration analyzer. Total RNA was reverse-transcribed into cDNA using the SuperScript III reverse transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, qPCR was performed using an ABI 7300 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR® Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sequences of reverse and forward primers for all of the genes analyzed were as follows: Skp2 (forward: ATGCCCCAATCTTGTCCATCT, reverse: CACCGACTGAGTGATAGGTGT); P27 (forward: AGGAGG AGATAGAAGCGCAGA, reverse: GTGCGGACTTGGTAC AGGT); Bcl-2 (forward: AGATGGGAACACTGGTGGAG, reverse: CTTCCCCAAAAGAAATGCAA); Bax (forward: AGGGTTTCATCCAGGATCGAGCA, reverse: CAGCTT CTTGGTGGACGCATC); Caspase-3 (forward: ACATCT CCCGGCGGCGGGCCGCGGA, reverse: CTTCTACAA CCGCCTCACAATAGCA); Caspase-9 (forward: AGTTGG CTACTCGCCATGGACGAAG, reverse: TTTGCTGCTTGC CTGTTAGTTCGCA); β-actin (forward: GACAGGATGCAG AAGGAGATTACT, reverse: TGATCCACATCTGCTGGA AGGT). The following thermocycling conditions were used for qPCR: 5 min at 95˚C; followed by 40 cycles of 10 sec at 95˚C, 20 sec at 58˚C, 20 sec at 72˚C and 15 sec at 95˚C; 60 sec at 60˚C; and final extension for 15 sec at 95˚C. All reactions

Table I. IC_{50} value of different contention of single LY294002 and ABT199 at different time points in K562, HL60 and KG1a cells.

	K562 cells		HL60 cells		KG1a cells	
IC_{50} value	LY294002 (μM)	ABT199 (nM)	LY294002 (μM)	ABT199 (nM)	LY294002 (μM)	ABT199 (nM)
24 h	1.637	23.666	3.373	1100.611	2.794	36.294
36 h	1.612	24.445	3.472	957.016	2.942	313.530
48 h	1.433	22.498	3.893	262.94	2.853	389.674
72 h	1.547	22.128	4.577	464.444	6.758	378.516

were performed in triplicate. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (41) and normalized to the internal reference gene β-actin.

Western blot analysis. Total protein was extracted from K562, HL60 and KG1a cells and mixed with pyrolysis liquid. Maximum power ultrasonic was used for cell crushing in the ice bath (3x10 sec). Protein concentrations were determined using the BCA protein quantitative method. Equivalent amounts of protein (25 μg) were separated via 12% SDS-PAGE and electro-transferred onto a PVDF membrane in 1X protein transfer membrane solution in ice water for 1.5 h. Following blocking in PBS supplemented with 5% skimmed dry milk at room temperature for 1 h, the membranes were incubated at 4˚C overnight with primary antibodies of Skp2 (monoclonal antibody, 1:500, bs-1096R, BIOSS), P27 (polyclonal antibody, 1:200, DF6090, Affinity Biosciences), Bcl2 (polyclonal antibody, 1:500, bs-0032R, BIOSS), Bax (polyclonal antibody, 1:500, bs-0127R, BIOSS), cleaved casepase-3 (polyclonal antibody, 1:500, bs-0081R, BIOSS), cleaved casepase-9 (polyclonal antibody, 1:500, bs-0049R, BIOSS), Procasepase-3 (monoclonal antibody, 1:500, sc-7272, Santa Cruz Biotechnology) and Procasepase-9 (monoclonal antibody, 1:500, sc-70506, Santa Cruz Biotechnology). Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies (1:5,000, ZB-2305, ZSGB-BIO) at room temperature for 1 h. Protein bands were visualized using enhanced ECL chemiluminescence reagents followed by exposure to X-ray film. The protocol was repeated three times by using ImageJ software.

Statistical analysis. Statistical analyses were performed using SPSS statistical software (SPSS, Inc.). Data are presented as the mean ± SD. ANOVA and Dunnett's post hoc test was used for comparison analysis between the two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Determination of optimal concentration and duration of LY294002 and ABT199 combination treatment. To identify the optimal concentration and duration of LY294002 and ABT199 combination treatment, the IC_{50} values of different concentrations of LY294002, ABT199 or LY294002 and ABT199 combination treatment at 24, 36, 48 and 72 h in K562, HL60 and KG1a cells were calculated. A synergistic effect was observed when the inhibitory rate of the combination treatment was greater than the sum of the inhibitory rates of the two single drugs.

In K562 cells (Table I), the IC_{50} value of LY294002 (1.433 μM) was the lowest following treatment for 48 h. At 48 h, the IC_{50} value of ABT199 was 22.498 nM. Therefore, the combination of LY294002 <1.433 μM and ABT199 <22.498 nM at 48 h was used as the screening criteria. The combination of 0.97 μM LY294002 and 18.222 nM ABT199 at 48 h was considered to be the optimal concentration and duration of drug combination action in K562 cells (Table II).

In HL60 cells (Table I), the IC_{50} value of ABT199 (262.94 nM) was the lowest after treatment for 48 h. At 48 h, the IC_{50} value of LY294002 was 3.893 μM. Therefore, the combination of LY294002 <3.893 μM and ABT199 <262.94 nM at 48 h was used as the screening criteria. The combination of 0.57 μM LY294002 and 22.476 nM ABT199 at 48 h was considered the optimal concentration and duration of drug combination action in HL60 cells (Table III).

In KG1a cells (Table I), the IC_{50} value of LY294002 (2.794 μM) was the lowest after treatment for 24 h. At 24 h, the IC_{50} value of ABT199 (36.294 nM) was also the lowest. Therefore, the combination of LY294002 <2.794 μM and ABT199 <36.294 nM at 24 h was used as the screening criteria. The combination of 0.97 μM LY294002 and 23.141 nM ABT199 at 24 h was considered to be the optimal concentration and duration of drug combination action in KG1a cells (Table IV).

RT-qPCR. To further investigate the effects of LY294002 and ABT199 combination treatment on K562, HL60 and KG1a cells at the molecular level, six cell cycle-related molecular markers [S-phase kinase associated protein 2 (Skp2), p27, Bcl2, Bax, cleaved caspase-3 and caspase-9] were evaluated. The primer sequences are shown in Table V. In K562 cells (Fig. 1), Skp2 and Bcl2 expression levels were significantly downregulated after LY294002 and ABT199 combination treatment. p27, Bax, cleaved caspase-3 and caspase-9 expression levels were markedly upregulated by single and combination treatment with LY294002 and ABT199. In both HL60 (Fig. 2) and KG1a (Fig. 3) cells, Skp2 and Bcl2 expression levels were significantly downregulated in the single ABT199 treatment group and the combined treatment group. p27, Bax, cleaved caspase-3 and caspase-9 expression levels were significantly upregulated in the single LY294002, single ABT199 and combined treatment groups.

Western blotting. To assess the protein expression levels of Skp2, P27, Bcl2, Bax, procaspase-3, procaspase-9, cleaved

Table II. IC_{50} value of different contention of LY294002 and ABT199 combination at different time points in K562 cells.

IC_{50} value	ABT199 (nM)			
LY294002 (μM)	24 h	36 h	48 h	72 h
0.5	23.285	25.654	25.230	69.294
0.57	23.162	26.747	40.195	127.106
0.97	25.122	25.184	18.222	32.125
1.5	25.339	25.339	23.048	21.069
2.5	25.347	25.704	28.156	18.440
5	24.202	25.731	77.801	23.194

Table III. IC_{50} value of different contention of LY294002 and ABT199 combination at different time points in HL60 cells.

IC_{50} value	ABT199 (nM)			
LY294002 (μM)	24 h	36 h	48 h	72 h
0.5	42.234	183.449	24.275	37.273
0.57	21.314	183.569	22.476	160.804
0.97	56.324	196.308	26.998	81.574
1.5	18.712	35.984	28.015	60.768
2.5	19.210	43.297	28.457	39.534
5	23.052	28.282	63.810	19.902

caspase-3 and caspase-9 following LY294002 and ABT199 combination treatment, western blotting was performed in K562 (Fig. 4), HL60 (Fig. 5) and KG1a (Fig. 6) cells. The results demonstrated that Skp2 and Bcl2 protein expression levels were significantly decreased in the single LY294002, single ABT199 and combined treatment groups in all three cell lines. The protein expression levels of p27 and Bax, the ratio of cleaved procaspase-3/procaspase-3 and cleaved procaspase-9/procaspase-9 were remarkably increased in the single LY294002, single ABT199 and combined treatment groups, and significantly higher compared with single drug treatment.

Discussion

LY294002 blocked the proliferation of primary AML blasts by inhibiting AKT-induced survival signaling pathways and induced cell death (42-44). In addition, LY294002 induced AML cell apoptosis (42). Treatment with LY294002 led to a dose-dependent decrease in the phosphorylation of AKT, mTOR, eukaryotic translation initiation factor 4E binding protein 1, ribosomal protein S6 kinase B1 and ribosomal protein S6, which was associated with reduced cell viability due to increased apoptosis (45). Zhou *et al* (32) and Manda-Handzlik *et al (* 36) reported that LY294002 in combination with conventional chemotherapeutic drugs increased the sensitivity of AML cells to apoptosis. ABT199 can impair mitochondrial respiration and energy production in human leukemia stem cells (20). Clinical trials have demonstrated that ABT199 is a promising drug for the treatment of hematopoietic malignancy and chronic lymphocytic leukemia (46-48). Roche *et al* (27) reported that ABT199 showed promising single-agent activity in samples derived from patients with AML. Several clinical trials of hypomethylator-based combinations (ABT199 + decitabine/azacytidine) have doubled the response rate, improving the survival of patients with AML (33). In addition, ABT199 and ONC212 combination treatment was highly synergistic in the AML xenograft model (49). At the molecular level, LY294002 and ABT199 combination treatment significantly decreased Skp2 and Bcl2 expression levels, but markedly increased p27, Bax, cleaved caspase-3 and caspase-9 expression levels in K562, HL-60 and KG1a cells. Skp2 is involved in leukemia cell proliferation and is associated with chronic myeloid leukemia (50,51). Kojima *et al* (43) and Park *et al* (44) reported that Skp2 expression was increased in leukemia and AML. The p27

Table IV. IC_{50} value of different concentrations of LY294002 and ABT199 combination at different time points in KG1a cells.

IC_{50} value	ABT199 (nM)			
LY294002 (μM)	24 h	36 h	48 h	72 h
0.5	39.457	228.364	106.779	130.807
0.57	24.414	167.608	208.388	24.812
0.97	23.141	52.986	198.294	16.557
1.5	32.621	26.569	104.024	41.357
2.5	23.782	21.719	75.506	15.062
5	25.547	17.115	39.875	16.435

Table V. Primer sequences used in RT-qPCR.

Target name	Primer	Sequences
β-actin	F	GACAGGATGCAGAAGGAGATTACT
	R	TGATCCACATCTGCTGGAAGGT
Skp2	F	ATGCCCCAATCTTGTCCATCT
	R	CACCGACTGAGTGATAGGTGT
P27	F	AGGAGGAGATAGAAGCGCAGA
	R	GTGCGGACTTGGTACAGGT
Bcl-2	F	AGATGGGAACACTGGTGGAG
	R	CTTCCCCAAAAGAAATGCAA
Bax	F	AGGGTTTCATCCAGGATCGAGCA
	R	CAGCTTCTTGGTGGACGCATC
Caspase-3	F	ACATCTCCCGGCGGCGGGCCGCGGA
	R	CTTCTACAACCGCCTCACAATAGCA
Caspase-9	F	AGTTGGCTACTCGCCATGGACGAAG
	R	TTTGCTGCTTGCCTGTTAGTTCGCA

F, forward; R, reverse.

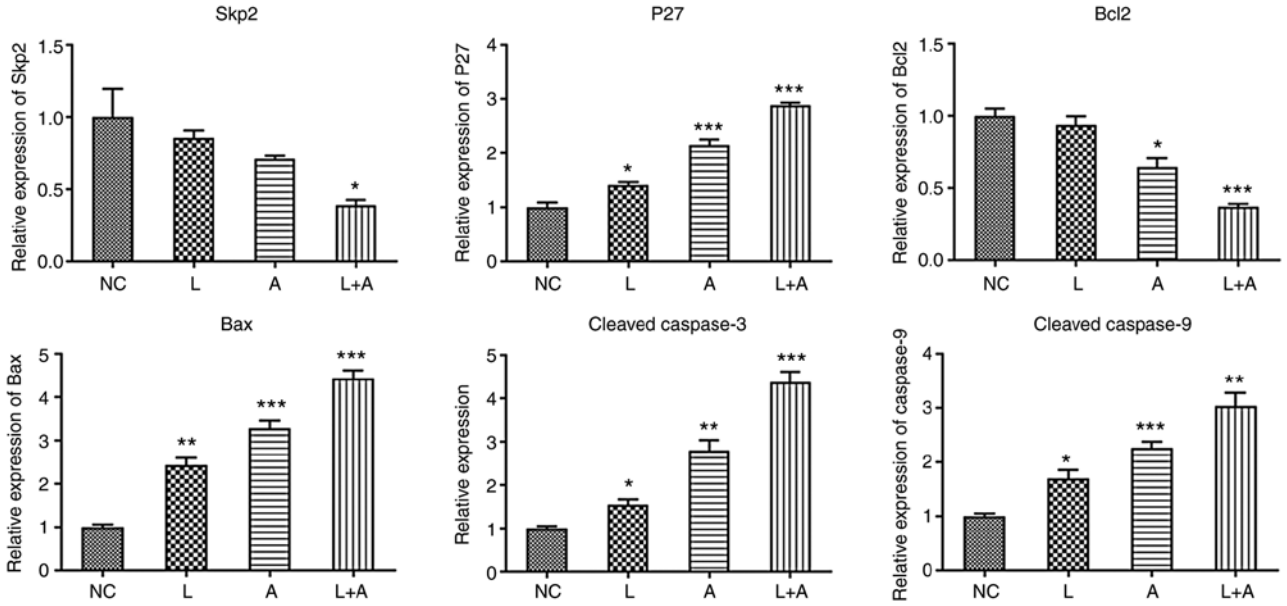

Figure 1. Skp2, p27, Bcl2, Bax, cleaved caspase-3 and cleaved caspase-9 mRNA expression levels in different drug treatment groups in K562 cells. *P<0.05, **P<0.01 and ***P<0.001. Skp2, S-phase kinase associated protein 2; NC, normal controls; L, LY294002; A, ABT199; L+A, LY294002+ ABT199.

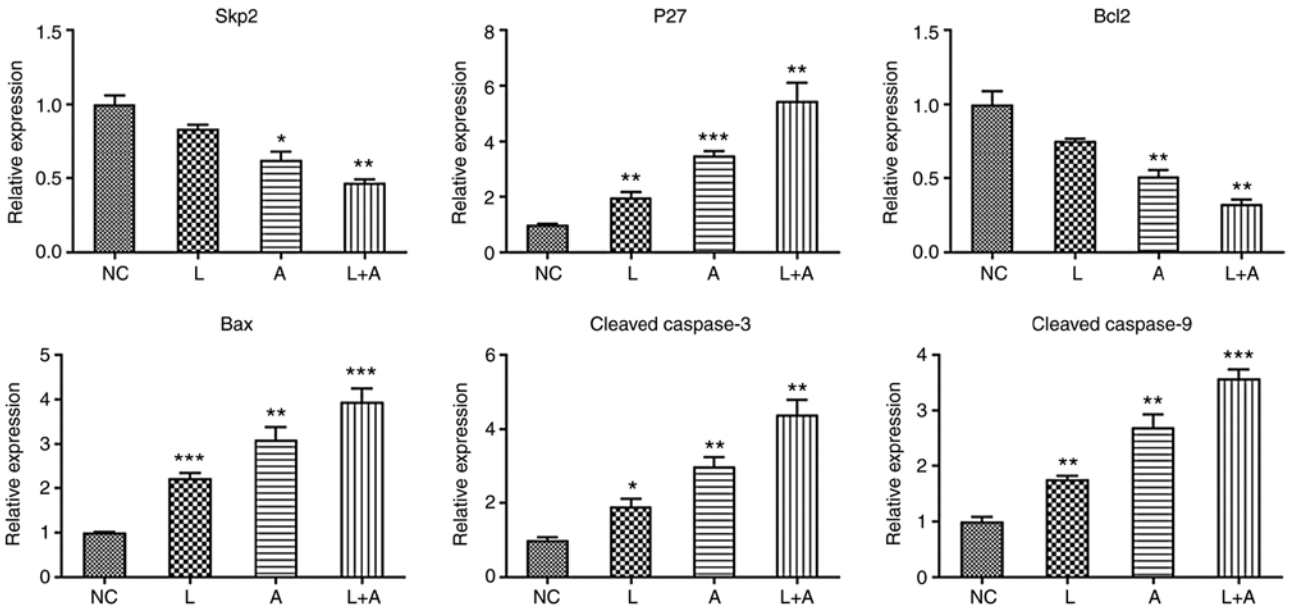

Figure 2. Skp2, p27, Bcl2, Bax, cleaved caspase-3 and cleaved caspase-9 mRNA expression levels in different drug treatment groups in HL60 cells. *P<0.05, **P<0.01 and ***P<0.001. Skp2, S-phase kinase associated protein 2; NC, normal controls; L, LY294002; A, ABT199; L+A, LY294002+ ABT199.

gene is located within a high incidence translocation region of leukemic chromosomes (52). p27 expression levels can serve as a prognostic reference to predict the outcomes of patients with pediatric acute lymphoblastic leukemia, particularly for disease recurrence (52). High p27 expression has a favorable prognostic impact in patients with AML (53). Bax is frequently associated with therapy resistance and is an attractive target for the development of anti-AML agents (3). It is reported that the apoptotic network of inactivation of BAX mediated resistance to BCL2 inhibition in AML (47). In human acute promyelocytic leukemia, cleaved caspase-3 induces apoptosis and decreases cell proliferation (54). During normal hematopoiesis, caspase-9 is not required for cell apoptosis (55). In AML, a mutation in caspase-9 has been identified (56). Furthermore, it has been demonstrated that caspase-9 serves a non-redundant role in the pathogenesis of T-therapy-related AML (57).

The present study indicated that LY294002 and ABT199 served a synergistic role in inhibiting the cell cycle, which suggested LY294002 and ABT199 combination treatment may serve as a novel therapeutic strategy for AML. However, the present study had a number of limitations. Future studies should use an animal model of AML to further investigate the effect of LY294002 and ABT199 combination treatment on cell apoptosis and the cell cycle in AML. Moreover, the functional study of BCR-ABL, sphingosine kinase (SphK)1

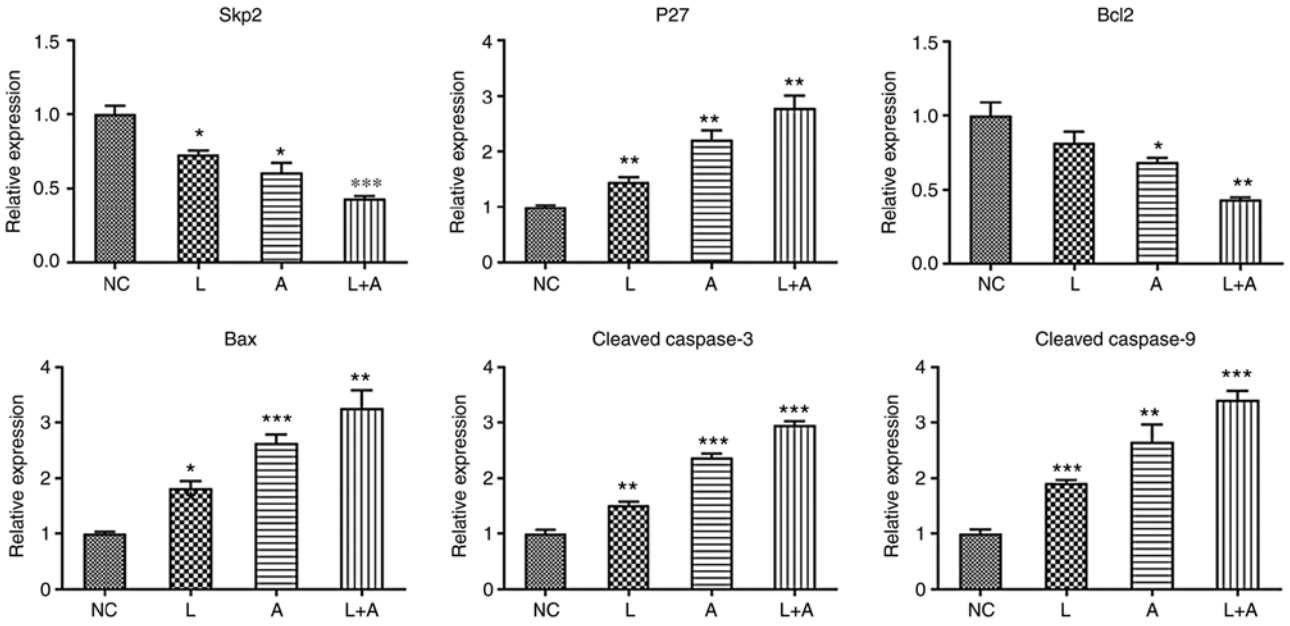

Figure 3. Skp2, p27, Bcl2, Bax, cleaved caspase-3 and cleaved caspase-9 mRNA expression levels in different drug treatment groups in KG1a cells. *P<0.05, **P<0.01 and ***P<0.001. Skp2, S-phase kinase associated protein 2; NC, normal controls; L, LY294002; A, ABT199; L+A, LY294002+ ABT199.

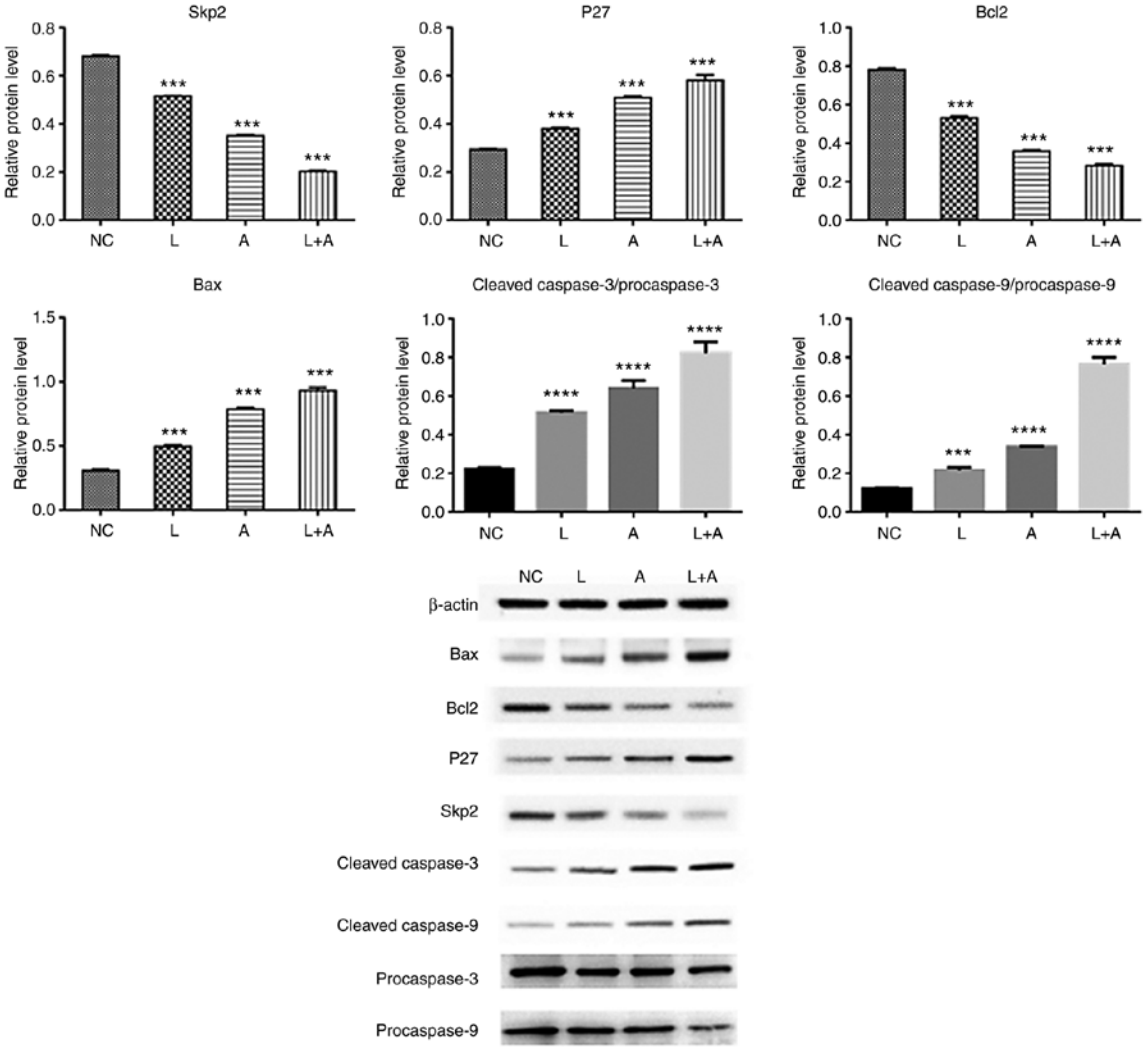

Figure 4. Skp2, p27, Bcl2 and Bax protein expression levels, and the ratio of cleaved procaspase-3/procaspase-3 and cleaved procaspase-9/procaspase-9 in different drug treatment groups in K562 cells. ***P<0.001; ****P<0.0001. Skp2, S-phase kinase associated protein 2; NC, normal controls; L, LY294002; A, ABT199; L+A, LY294002+ ABT199.

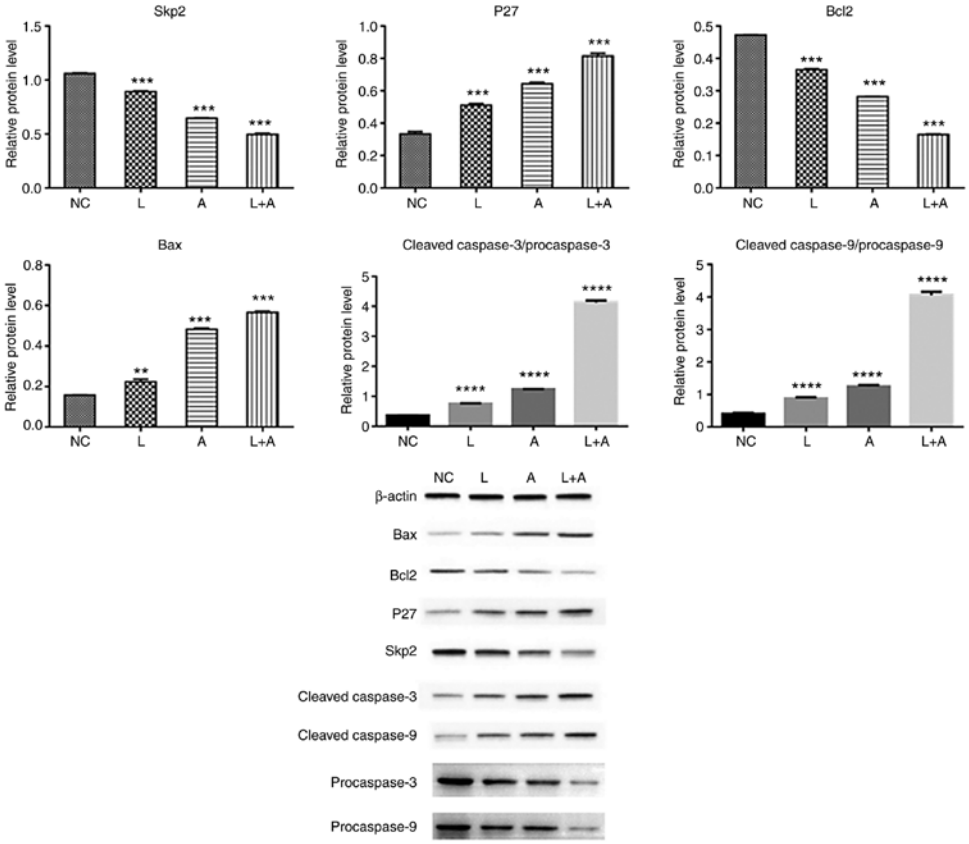

Figure 5. Skp2, p27, Bcl2 and Bax protein expression levels, and the ratio of cleaved procaspase-3/procaspase-3 and cleaved procaspase-9/procaspase-9 in different drug treatment groups in HL60 cells. **P<0.01 and ***P<0.001; ****P<0.0001. Skp2, S-phase kinase associated protein 2; NC, normal controls; L, LY294002; A, ABT199; L+A, LY294002+ ABT199.

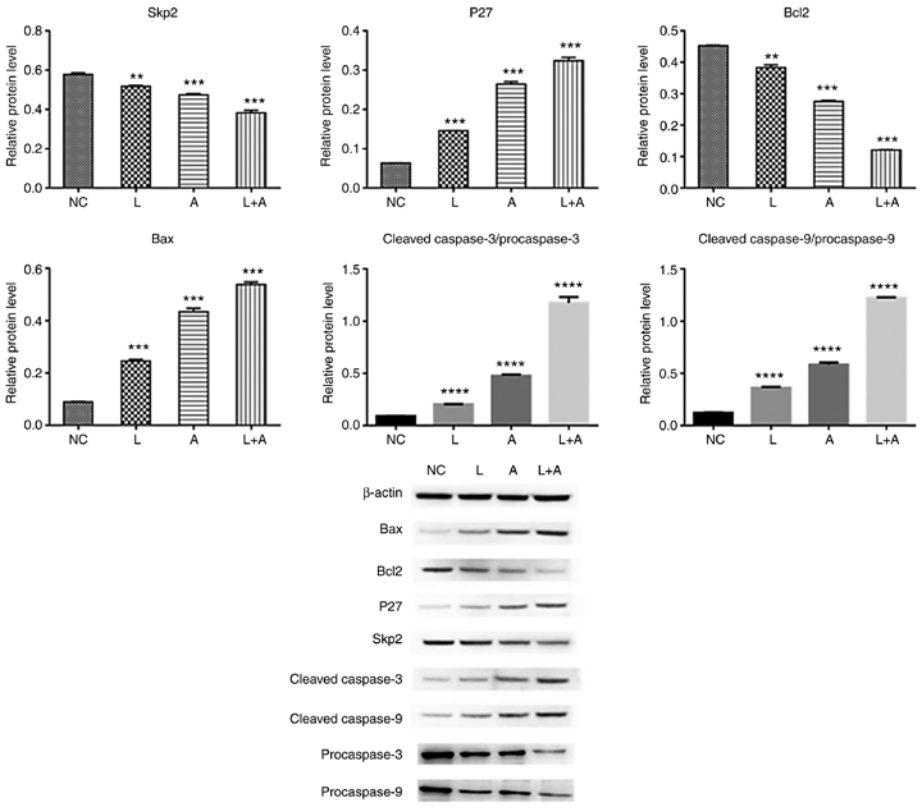

Figure 6. Skp2, p27, Bcl2 and Bax protein expression levels, and the ratio of cleaved procaspase-3/procaspase-3 and cleaved procaspase-9/procaspase-9 in different drug treatment groups in KG1a cells. **P<0.01 and ***P<0.001; ****P<0.0001. Skp2, S-phase kinase associated protein 2; NC, normal controls; L, LY294002; A, ABT199; L+A, LY294002+ ABT199.

and SphK2 in all cell lines should be conducted in future studies.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YHG and WJW conceived and designed the study. YHG was responsible for sample collection and WJW provided administrative support. YBG and LLZ collected and collated data and conducted analysis and interpretation thereof. JL and YLY contributed to data analysis and interpretation. YBG and YLY wrote and revised the manuscript. All authors approved of the final manuscript. In addition, the authenticity of all the raw data was assessed by YBG and YLY to ensure its legitimacy.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Conflicts of interest

The authors declare that they have no competing interests.

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