

# Cordycepin induces apoptosis of human acute monocytic leukemia cells via downregulation of the ERK/Akt signaling pathway

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**Abstract.** The aim of the present study was to examine the apoptotic effect of cordycepin (COR) on human THP-1 acute monocytic leukemia cells. THP-1 cells were exposed to different concentrations of COR for 24, 48, 72 or 96 h. The cell viability and apoptotic rate were analyzed. The gene expression of Akt1, Akt2, Akt3, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) were assessed by reverse-transcription quantitative PCR. Western blot analysis was used to detect the protein levels of phosphorylated (p)-Akt, p-extracellular signal-regulated kinase (ERK) and cleaved caspase-3. It was found that the viability of THP-1 cells was inhibited by COR in a dose- and time-dependent manner. After treatment with 200  $\mu$ M COR for 24 h, the percentage of apoptotic cells was significantly increased. COR also downregulated the levels of Bcl-2, Akt1, Akt2 and Akt3, and elevated the expression of Bax. The protein levels of p-Akt and p-ERK were suppressed and cleaved caspase-3 was increased after treatment of COR. In conclusion, COR was found to induce apoptosis of THP-1 acute monocytic leukemia cells through downregulation of ERK/Akt signaling.

## Introduction

Acute myeloid leukemia (AML) is a clonal disorder, which comprises a group of clonal malignant diseases. The leukemia

cells originate from the bone marrow, giving rise to an accumulation of abnormal immature myeloid cells in the bone marrow and blood (1,2). AML is one of the most common leukemia types. Over 50% of patients diagnosed with AML are >65 years of age (3). The main therapeutic strategies for AML patients are aggressive chemotherapeutic regimens and hematopoietic stem cell transplantation, and ~40% of AML patients receive chemotherapy within 3 months after diagnosis (4,5). In spite of intensive chemotherapy being able to achieve complete remission in most AML patients, the overall survival rate remains poor and the therapeutic process is usually associated with serious adverse events (6). However, AML comprises the following 8 types: M<sub>0</sub>, minimally differentiated AML; M<sub>1</sub>, AML without maturation; M<sub>2</sub>, AML with maturation; M<sub>3</sub>, acute pro-myelocytic leukemia; M<sub>4</sub>, acute myelomonocytic leukemia; M<sub>5</sub>, acute monocytic leukemia (AMoL); M<sub>6</sub>, erythroleukemia; and M<sub>7</sub>, acute megakaryoblastic leukemia. And there is no treatment plan for any of the AML type (7-10). The major objective of the present study was to identify a drug for curing AMoL.

The development of AMoL is a multistep and multifactorial process. The neoplastic cells are most frequently derived from white blood cells, have a high rate of marrow infiltration and high bad karyotype ratio. As AMoL is insensitive to numerous chemotherapy regimens, affected patients have a low remission rate and short lifetime (11-16). Therefore, a novel and effective drug is urgently required.

In recent years, bioactive natural products have emerged and received a considerable amount of attention from researchers. *Cordyceps militaris* is a traditional Chinese medicinal plant, which has been widely used for a long time. Recent studies have demonstrated that biologically active components isolated from *Cordyceps* species have various pharmacological effects (17,18). Cordycepin (COR) is one of the most widely studied active components of *Cordyceps militaris* and has diverse biological functions, such as anti-tumor (19), anti-invasive (20) and anti-inflammatory effects (21). However, the effects and potential mechanisms of COR in AMoL have largely remained to be elucidated. Therefore, the purpose of the present study was to evaluate the anti-cancer effect of COR on AMoL and investigate the potential underlying mechanisms. The present findings suggested that COR induces apoptosis of the THP-1 AMoL cell line via deactivating Akt and

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extracellular signal-regulated kinase (ERK) and upregulating the expression of cleaved caspase-3 indicating that COR may be a potential therapeutic drug for AMoL.

## Materials and methods

**Reagents.** COR and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). COR was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA), and the proportion of DMSO in the culture medium was <0.05%. COR was stored at -20°C. Primary antibodies against phosphorylated (p)-ERK (Cat no. 4348S), total (t)-ERK (Cat no. 4695S), p-Akt (Cat no. 4060S), t-Akt (Cat no. 4685S), cleaved caspase-3 (Cat no. 1050S),  $\beta$ -actin (Cat no. 4970S) as well as Anti-rabbit Immunoglobulin G (IgG) secondary antibodies (Cat no. 5151S) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

**Cell culture.** THP-1 AMoL cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). THP-1 cells were cultured in RPMI 1640 medium (Hyclone; GE Healthcare, Little Chalfont, UK) containing 10% fetal bovine serum (Hyclone; GE Healthcare), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Hyclone; GE Healthcare) in an incubator at 37°C with 5% CO<sub>2</sub>. All cells we used at a passage of <20.

**Measurement of cell viability.** A Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used to assess the potential therapeutic effect of COR. For measurement of cell proliferation, THP-1 AMoL cells were seeded into each well of a 96-well plate at a density of 20,000 cells/well in 100  $\mu$ l culture media containing 100 nM PMA to induce adherence, and treated with 0, 25, 50, 100, 150, 200  $\mu$ mol/l COR for 96 h after adherence (22). Following treatment with COR for 24, 48, 72 and 96 h, 100  $\mu$ l culture media and 10  $\mu$ l CCK-8 solution were added to each well, followed by incubation at 37°C for 150 min. The optical density (OD) value at 450 nm was determined using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Each experimental condition was repeated in three wells. The cell viability relative to that in the 0  $\mu$ mol/l COR group (control) was calculated using the following equation: Cell viability (% of control group) =  $\text{OD}_{\text{drug-treated group}} / \text{OD}_{\text{control group}}$ .

**Apoptosis assay.** THP-1 cells were incubated with various concentrations of COR for 24 h. Subsequently, the cells were washed twice with cold PBS and harvested. The cells were re-suspended in 1X Annexin-binding buffer, and stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA). The apoptotic rate was measured by flow cytometry using a BD FACSCalibur™ analyzer (BD Biosciences, Franklin Lakes, NJ, USA). The total apoptotic rate of cells was considered to be the early apoptotic rate (lower right quadrant in the PI vs. FITC dot plot) plus the late apoptotic rate (upper right quadrant in the dot plot).

**RNA isolation and reverse-transcription quantitative polymerase chain reaction (RT-qPCR).** THP-1 cells were

cultured in complete medium in the presence of various concentrations of COR for 24 h. The total RNA of the THP-1 cells was then isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's instructions. For first-strand complementary DNA (cDNA) synthesis, RT was performed using the 1st Strand cDNA Synthesis kit (Takara Bio Inc., Dalian, China). The relative gene expression was determined by real-time PCR using the SYBR Premix Ex Taq kit (Takara Bio, Inc.) with the ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The real-time PCR thermocycling conditions were as follows: pre-degeneration at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 34 sec, and the dissociation stage was 34 sec at 95°C. The primers were designed and selected using the Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Gene expression was quantified using the 2<sup>- $\Delta\Delta$ Cq</sup> method (23).  $\beta$ -actin was used as the internal control. The primer sequences are listed in Table I.

**Western blot analysis.** THP-1 cells were treated as for the RT-qPCR analysis and then lysed with radioimmunoprecipitation assay buffer (Upstate Biotechnology, Inc., Lake Placid, NY, USA) supplemented with a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). For western blot analysis, protein concentration was quantified using the BCA Protein Assay kit (Thermo Fisher Scientific, Inc.; catalogue no. 23250) according to manufacturer protocol. Samples were adjusted to the same protein concentration prior to loading (30  $\mu$ g each sample) and separated by 10% SDS-PAGE for p-Akt, t-Akt, p-ERK, t-ERK and  $\beta$ -actin and 12.5% SDS-PAGE for cleaved caspase-3. Subsequently, proteins were transferred onto a nitrocellulose membrane (Merck Millipore, Billerica, MA, USA). Antibodies against p-Akt, t-Akt, p-ERK, t-ERK and cleaved caspase-3 were applied at the dilutions recommended by the manufacturer (1:1,000) at 4°C overnight.  $\beta$ -actin (1:1,000) was used as a loading control. After 37 min washes in Tris-buffered saline containing Tween-20 (TBST), the membranes were incubated with anti-rabbit second antibody (1:15,000; Cell Signalling Technology, Inc) for 1 h at room temperature. The target proteins were detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). The quantification of the bands was analyzed by the software of Odyssey Infrared Imaging System (Version 3.0).

**Statistical analysis.** All of the experiments were repeated 3 times. Values are expressed as the mean  $\pm$  standard deviation. Statistical analysis was performed by one-way analysis of variance, followed by Duncan's post-hoc test using SPSS 19.0 (International Business Machines, Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**COR inhibits THP-1 cell proliferation.** To assess the effects of COR on the proliferation of THP-1 cells, a CCK-8 assay using was performed. The inhibitory effect of COR on the proliferation of THP-1 cells was strengthened with

Table I. Sequences of primers used for polymerase chain reaction.

Gene	Direction	Primer sequence (5'-3')
Akt1	Forward	GAAGGACGGGAGCAGGC
	Reverse	CTCACGCGCTCCTCTCAG
Akt2	Forward	TGCCACCATGAATGAGGTGAA
	Reverse	GTACCCAATGAAGGAGCCGT
Akt3	Forward	TTTCTCCAAGTTGGGGGCTC
	Reverse	CCCCTCTTCTGAACCCAACC
Bcl-2	Forward	TTTGTGGAAGTGTACGGCCC
	Reverse	GTTGACTTCACTTGTGGCCC
Bax	Forward	AGCAGATCATGAAGACAGGGG
	Reverse	TGCTCGATCCTGGATGAAACC
$\beta$ -actin	Forward	CTCACCATGGATGATGATATCGC
	Reverse	AGGAATCCTTCTGACCCATGC

Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

the increase of the COR concentration after 24, 48, 72 and 96 h of treatment (Fig. 1). In conclusion, COR inhibited the proliferation of THP-1 cells in a dose- and time-dependent manner.

*COR concentration-dependently induces apoptosis in THP-1 cells.* As presented in Fig. 2, flow cytometric analysis was employed to detect COR-induced apoptosis. Different concentrations of COR increased the total apoptotic rate in a dose-dependent manner (3.35% in the control vs. 11.48, 13.67 and 24.99% after treatment with COR at 100, 150 and 200  $\mu$ M, respectively;  $P < 0.05$ ; Fig. 2A-G). Quantitative results on the early and late apoptotic rates were consistent with this phenomenon (Fig. 2H-I). To conclude, COR was found to exert its inhibitory function on THP-1 cells by induction of apoptosis, resulting in cell death.

*COR regulates apoptosis- and survival-associated gene expression in THP-1 cells.* To investigate the in-depth molecular mechanisms of the inhibitory effect of COR on THP-1 human monocytic leukemia cells, RT-qPCR was performed to examine its impact on the expression of apoptosis-associated genes. It was found that the expression levels of Bcl-2 were reduced in COR-treated THP-1 cells in a dose-dependent manner, while the expression of Bax was upregulated (Fig. 3). In addition, Akt signaling is crucial for the initiation and progression of AML and also regulates a number of downstream targets responsible for cell survival and proliferation (24,25). Therefore, the current study detected the mRNA level of three isoforms (Akt1, Akt2 and Akt3) of Akt. The cell survival-associated genes Akt1, Akt2 and Akt3 were downregulated after COR treatment (Fig. 4). Therefore, the phosphorylation of Akt was detected by western blot analysis based on the levels of mRNA (Fig. 4). These results indicated that these genes are potentially involved in signaling pathways downstream of those targeted by COR in monocytic leukemia cells.

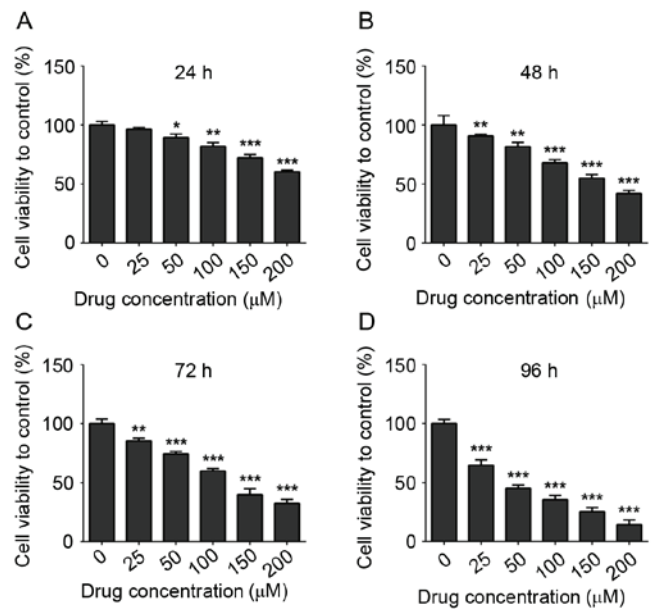


Figure 1. Anti-proliferative effects of COR on THP-1 cells. (A-D) A Cell Counting Kit-8 assay demonstrated the anti-proliferative effects of COR on THP-1 cells after treatment for 24, 48, 72 and 96 h. Values are expressed as the mean  $\pm$  standard error. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  vs. untreated group. COR, cordycepin.

*Akt and ERK are potential downstream targets of COR.* To evaluate the potential involvement of the signal transduction pathways and the mechanisms of the effects of COR on THP-1 cells, the expression of various signaling proteins was determined in THP-1 cells by western blot analysis following treatment with different concentrations of COR. The results demonstrated that COR inhibited the protein expression of p-Akt and p-ERK in a concentration-dependent manner significantly ( $P < 0.05$ ; Fig. 5A-D). However, cleaved caspase-3 was significantly activated by increasing concentrations of COR ( $P < 0.05$ ; Fig. 5E and F). Taken together, these results suggested that Akt and ERK are potential downstream targets of COR in THP-1 cells, and that apoptosis is induced via associated signaling pathways.

## Discussion

The major challenge in the treatment of AML is the high failure and relapse rate due to drug resistance. Application of novel drug treatments is one of the therapeutic approaches for patients with resistance to standard therapies. Clinical evaluation of potential effective chemotherapeutic drugs may provide novel treatments, which may improve the prospects of refractory leukemia patients. AMoL is a rare but distinct disease entity, which is most remarkable due to its clinical course. Cytogenetic characterization is essential for the diagnosis and determination of the prognosis of patients (26-28). There is an urgent requirement to identify less toxic and more effective treatments, and an increasing amount of research has focused on the application of natural products in AMoL treatment.

COR was previously reported to have anti-tumor (29,30), anti-inflammatory (31) and anti-oxidant (32) activities. However, the potential role and mechanisms of COR in the treatment of AMoL have remained to be determined. The

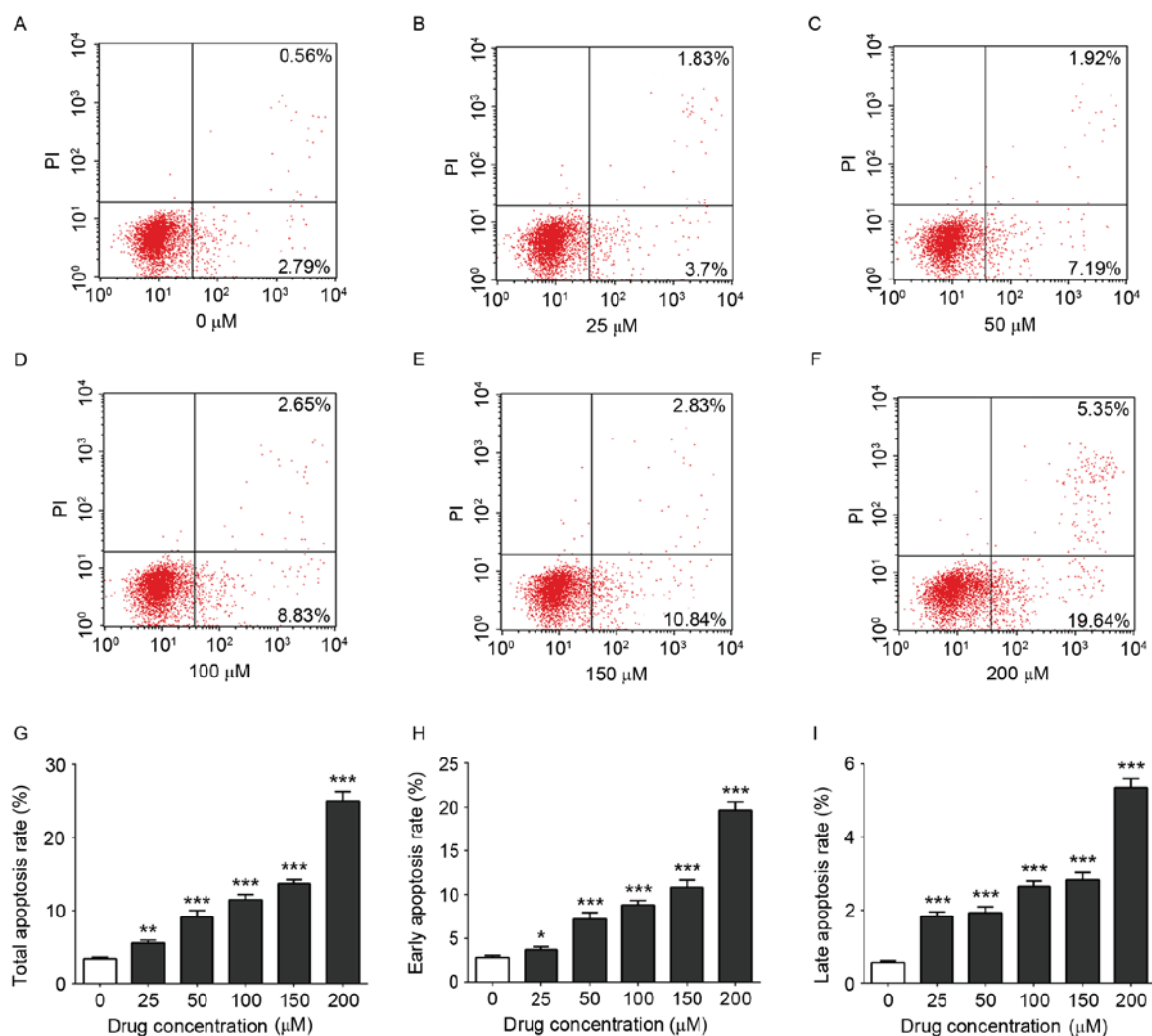


Figure 2. Apoptosis of THP-1 cells following COR treatment. (A-F) Flow cytometry-based assessment of apoptosis in THP-1 cells following treatment with 0, 25, 50, 100, 150, 200 μM of COR. Annexin V-fluorescein isothiocyanate is displayed on the x-axis. (G-I) Quantitative analysis of total, early and late apoptotic rate. Values are expressed as the mean ± standard error. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 vs. untreated group. COR, cordycepin; PI, propidium iodide.

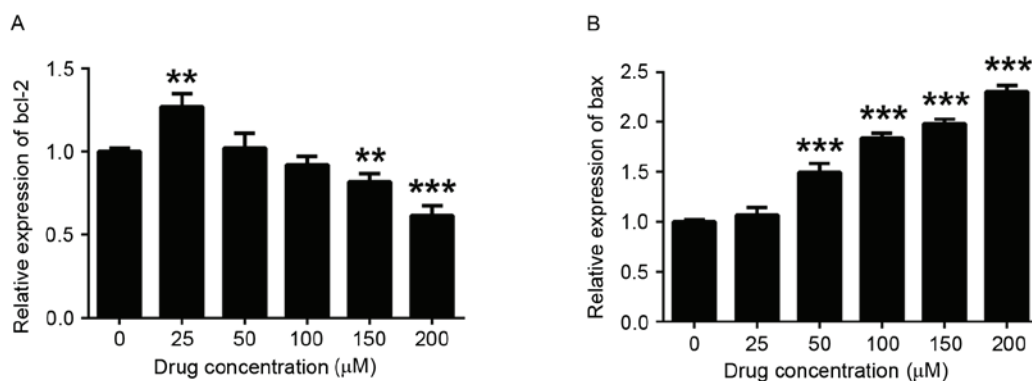


Figure 3. Apoptosis-associated gene expression in THP-1 cells after COR treatment. Reverse-transcription quantitative polymerase chain reaction analysis was employed to detect the expression of (A) Bcl-2 and (B) Bax in COR-treated THP-1 cells. Values are expressed as the mean ± standard error. \*\*P<0.01; \*\*\*P<0.001 vs. untreated group. COR, cordycepin; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

aim of the present study was to examine whether COR had anti-cancer activity against AMoL. The results demonstrated that COR exerted a marked inhibitory effect on the THP-1 AMoL cell line *in vitro*. COR inhibited the spontaneous growth

of THP-1 cells in a dose- and time-dependent manner. To further investigate the mechanisms of the anti-AMoL activity of COR, the effects of COR on apoptosis and activation of Akt/ERK survival signaling as well as Bcl-2/Bax/caspase-3

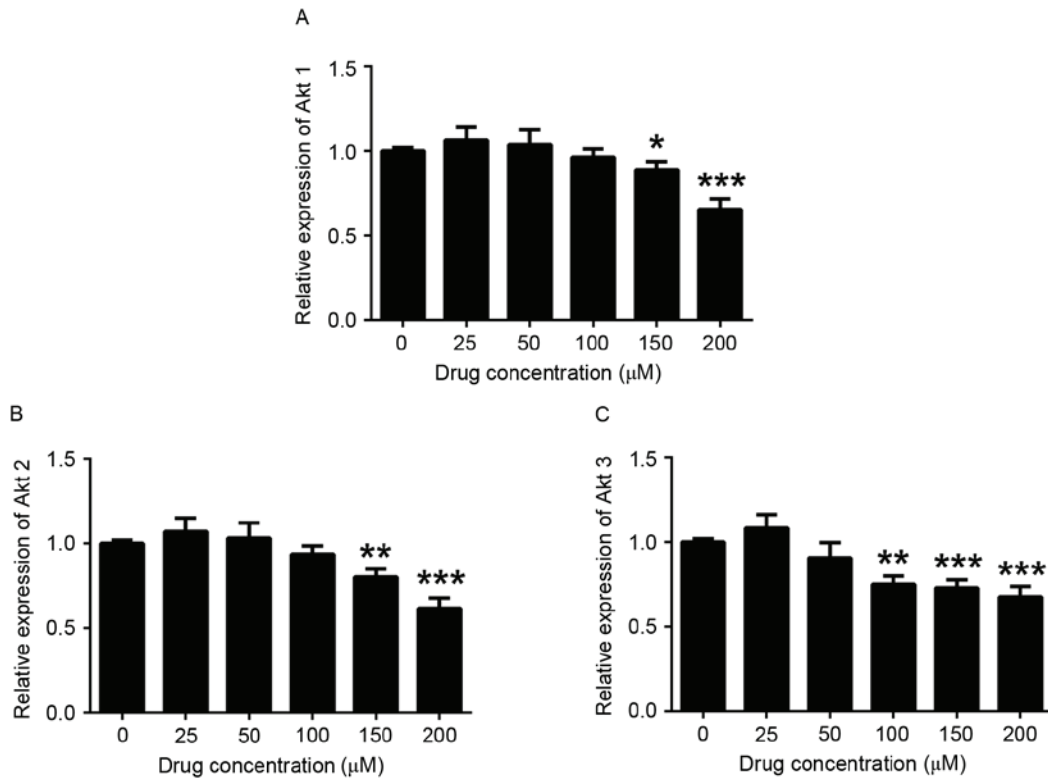


Figure 4. Cell survival-associated gene expression in THP-1 cells after COR treatment. Reverse-transcription quantitative polymerase chain reaction analysis was employed to detect the expression of (A) Akt1, (B) Akt2 and (C) Akt3 in COR-treated THP-1 cells. Values are expressed as the mean ± standard error. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 vs. untreated group. COR, cordycepin.

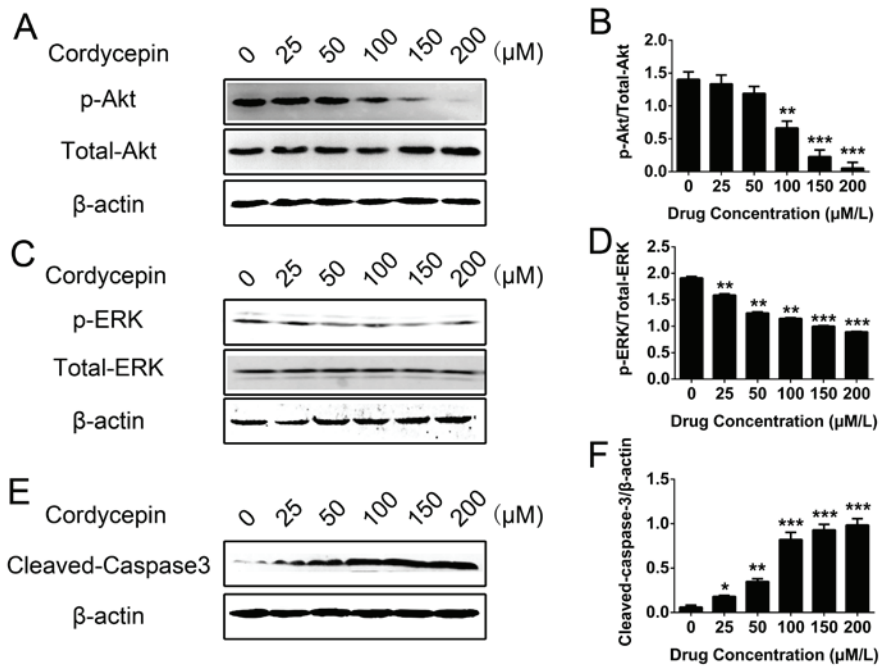


Figure 5. Western blot analysis of p/total-Akt, p-ERK/total-ERK and cleaved caspase-3 levels following apoptosis induction by COR in THP-1 cells. (A-C) Representative western blot displaying the levels of (A) p/total-Akt, (B) quantified, (C) p-ERK/total-ERK, (D) quantifies, (E) cleaved caspase-3 and, (F) quantified in THP-1 cells following COR treatment. Values were normalized to β-actin. Values are expressed as the mean ± standard error. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 vs. untreated group. COR, cordycepin; p-ERK, phosphorylated extracellular signal-regulated kinase.

apoptotic pathways in THP-1 cells were assessed. It was revealed that COR induced apoptosis by inhibiting Akt/ERK

signaling and activating caspase-3 following disturbance of the balance of the Bcl-2/Bax axis.

Leukemia cells often display continued activation of survival and signaling pathways, such as Akt and ERK, due to gene mutations, including rearrangements and chromosome translocation, and survival signaling pathways have an important role in the proliferation, tumorigenesis, evasion of apoptosis and drug resistance (33-36). The more survival signaling pathways are constitutively active in AML, the poorer the prognosis. The results of the present study demonstrated that in the THP-1 AMoL cell line, Akt and ERK signal transduction pathways were simultaneously suppressed. The apoptotic protein caspase-3 was activated by downregulation of the Bcl-2/Bax ratio after COR treatment. It was speculated that inhibition of the activation of the Akt/ERK pathways and promotion of the activation of caspase-3 by COR may produce an enhanced response in anti-AMoL therapy. Compared with numerous specific inhibitors, which have a single target for the treatment of leukemia, COR may be more effective, as drug resistance frequently emerges due to hyperactivation of alternative signaling pathways under the treatment with a drug that has a single target. As Akt, ERK and caspase-3 signaling are all interconnected and are not separate pathway entities (37) and COR exhibited characteristics of a multi-targeted therapeutic, it may be deduced that it rarely causes resistance in AMoL, that its efficacy may be higher and that the response duration may be longer.

Based on the above, the role of COR in restricting the proliferation and inducing apoptosis of AMoL cells was indicated. COR was found to indirectly or directly affect the viability of AMoL cells via Akt/ERK survival signaling and caspase-associated apoptosis signaling, which are key controllers of cell survival and apoptosis (38-41). However, further research should be performed based on this preliminary research. First, the in-depth molecular mechanisms underlying the COR-mediated inhibition of cell survival and induction of apoptosis-associated signaling pathways should be elucidated. Furthermore, animal experiments should be performed to verify the therapeutic effect of COR *in vivo*. Finally, the efficacy of COR in patients may be assessed.

In conclusion, the present study revealed that COR was efficacious against AMoL *in vitro*. Furthermore, COR treatment led to a decrease of p-Akt and p-ERK, while increasing the levels of cleaved caspase-3 via increasing the Bcl-2/Bax ratio. These are likely to be downstream mechanisms, by which COR exerts its inhibitory effects in AMoL. However, further study is required to fully elucidate the underlying mechanisms.

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