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Lycorine induces apoptosis and down-regulation of Mcl-1 in human leukemia cells

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ABSTRACT

Lycorine is an alkaloid isolated from the bulb of the Amaryllidaceae Lycoris. Here, we report that treatment with lycorine resulted in survival inhibition and apoptosis induction in human leukemia cell lines. Lycorine induced apoptosis in human leukemia cells via intrinsic mitochondria pathway and caused a rapid-turnover of protein level of Mcl-1 which occurred before caspases activation. Furthermore, pronounced apoptosis accompanied by the down-regulation of Mcl-1 was also observed in blasts from patients with acute myeloid leukemia. Our findings suggest that lycorine may be a good candidate therapeutic agent against leukemia in worth of further evaluation.

1. Introduction

Acute myeloid leukemia (AML) represents a group of aggressive hematological malignancies. Currently, AML patients are treated mainly by conventional chemotherapy combined with cytarabine or daunorubicin. While this therapy may induce complete remissions in 60–80% of young and 40–55% of elderly adult patients, long-term survivors among patients with complete remissions are few [1]. Thus, the development of novel chemical agents that are more effective in the selective killing of AML cells retains high priorities in leukemia research.

A key mechanism by which antileukemia agents kill leukemia cells is to activate the apoptosis pathways of the cells [2]. Apoptosis can be initiated through either the death receptor or the mitochondrial pathway. Both pathways are executed and regulated by Bcl-2 family of proteins [3]. One member of this family is myeloid cell leukemia-1 protein or Mcl-1, which is a pro-survival member. Mcl-1 was originally discovered as an early induced gene during the differentiation of the myeloid cell line ML-1 [4]. More recent studies have suggested that Mcl-1 may play an important survival role in a variety of tumor cells. First of all, Mcl-1 has been shown to be highly expressed in a variety of cancers including certain forms of leukemia [5]. Secondly, the targeted down-regulation of Mcl-1 by SiRNA triggers apoptosis in leukemia cells, while enhanced Mcl-1 expression contributes to a malignant phenotype in certain tumor cells [6,7]. More relevantly, Mcl-1 level has been shown to be elevated at the time of leukemia recurrence after chemotherapy in AML patients [8]. It has also been found that the down-regulation of Mcl-1 potentiates histone deacetylase inhibitor (HDACi)-induced apoptosis [9]. These together suggest that Mcl-1 may serve as a molecular target in antitumor therapy. One focus of intense interest is to develop treatments that can diminish the cellular level of the Mcl-1 protein.

In this work, we investigate the cytotoxic effects of the drug compound lycorine (Fig. 1) on human leukemia cell lines and the possible involvements of Mcl-1 in such effects. Lycorine is an active alkaloid isolated from Lycoris. It possesses various biological effects including antitumor [10], antiviral [11], antimalarial [12], antiinflammation [13]. It may inhibit the enzyme acetylcholinesterase [14] and the ascorbic acid biosynthesis [15]. The drug is

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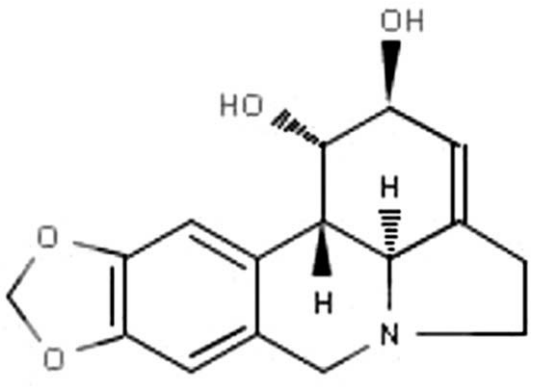

Fig. 1. Chemical structure of lycorine.

clinically used in Russian as an expectorant to treat chronic and acute inflammatory processes in lungs and bronchial diseases [16,17]. Several studies have shown that lycorine has selective cell type-dependent cell-killing effects on tumor cells. It inhibits the growth of leukemia Molt 4 and cervical HeLa cells heavily but has weak effects on hepatoma HepaG2 cells [10,18]. More recently, it has been shown to induce apoptosis through regulation of the cell cycle in leukemia HL-60 cells and multiplemeyloma cell line KM3 [19,20]. Another recent study reported that lycorine exhibits anti-tumor activity against HL-60 cells in SCID mice [21]. So far, there has been no reported studies demonstrating and elucidating the mechanism of cytotoxicity of lycorine against leukemia cells. Our present studies demonstrate that lycorine is potent in inhibiting the growth and inducing the apoptosis in human leukemia cells. At the molecular level, lycorine causes a rapid-turnover of protein levels of Mcl-1. These results suggest that lycorine may be a good candidate therapeutic agent against leukemia.

2. Materials and methods

2.1. Reagents

Lycorine ($C_{16}H_{17}NO_4HCl$, $M_W = 323.77$) (Fig. 1) and 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium (MTT) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Boc-D-fmk and MG-132 were purchased from Calbiochem, San Diego, CA, USA. All reagents were prepared and used as recommended by their suppliers.

2.2. Cell Lines and cell culture

K562, U937, and HL-60 cell lines were kindly provided by Dr. Jun Yin (Shantou University, Shantou, China). 6T-CEM cell line was purchased from Cell Bank in Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China. The imatinib-resistant K562/G01 cell line was purchased from Institute of Hematology, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China [22]. Fresh peripheral blood mononuclear cells (PBMNCs) from five healthy subjects and bone marrow specimens from three AML patients were collected after their informed consent had been obtained. Mononuclear cells were separated by Ficoll–Hipaque density sedimentation. The percentage of blasts for patients was >80%. Cells were cultured separately in RPMI 1640 supplemented with penicillin, streptomycin, and 10% FBS. K562/G01 cells were maintained in RPMI1640 containing or lacking 4 μM of imatinib. Cells were collected at a concentration of 1×10^5 cells/ml, to which were added the designated drugs and maintained in a 37 °C, 5% CO_2, fully humidified incubator for the indicated time.

2.3. MTT assay

Cells were treated with different concentrations of lycorine. At certain times after treatment initiation, cell viability was estimated by the modified MTT-assay described previously [23]. Briefly, 10 μl of MTT solution (5 mg/ml in ddH_2O) was added to each well. Plates were then incubated for 4 h at 37 °C. Intracellular formazan crystals were dissolved by addition of 100 μl of isopropanol with 0.04 N HCl to each well, until the solution turned purple and absorbance analyzed in an enzyme-linked immunosorbent assay (ELISA) plate reader at 490 nm. Rate of inhibition was calculated by using the equation: Rate of inhibition = $(Ac - At)/Ac \times 100$, where At and Ac represent the absorbance in treated and control cultures, respectively. IC_{50}, the drug concentration causing a 50% decrease in cell survival, was determined by interpolation from dose–response curves.

2.4. Annexin V-FITC/propidium iodine FACS

Apoptosis of cells exposed to lycorine for 24 h was determined by flow cytometry using a commercially available Annexin V-FITC/propidium iodine apoptosis detection kit (KeyGen Biotech Co., Ltd., NanJing, China). After drug treatment, cells were collected and washed twice in ice cold PBS and resuspended in 500 μl of binding buffer at 1×10^5 cells/ml and incubated with 1 μl of AnnexinV/FITC and 5 μl of propidium iodine in the dark for 15 min at room temperature. Finally, samples were analyzed by flow cytometry and evaluated based on the percentage of early apoptotic cells for AnnexinV positive and PI negative.

2.5. DNA fragmentation assay

DNA fragmentation was analyzed after the extraction of DNA from cells exposed to 10 μM of lycorine for 24 h using Apoptotic DNA ladder kit (Applygen Technologies Inc., Beijing, China). The DNA was separated on a 1.5% agarose gel and visualized under UV light by ethidium bromide staining.

2.6. Cytochrome C release assay

Assay kits for cytochrome *C* release apoptosis (Calbiochem, San Diego, CA, USA) was used to assess the release of cytochrome *C* from mitochondria to cytosol. Briefly, cell samples were harvested and washed once with ice-cold phosphate-buffered saline (PBS) by centrifugation at 600g for 5 min at 4 °C. Cell pellets were resuspended in cytosol extraction buffer and Incubated on ice for 10 min

and homogenized in an ice-cold tissue grinder for 30 passes. Homogenate was centrifuged at 700g for 10 min at 4 °C and supernatant was centrifuged at 10,000g for 30 min at 4 °C. Supernatant was harvested as cytosolic fraction. The pellets were resuspended in mitochondrial extraction buffer and saved as mitochondrial fraction. Ten micrograms of each cytosolic and mitochondrial fraction was loaded on a 12% SDS–PAGE for standard procedure of Western blot.

2.7. Western blot analysis

A modified method as previously described was used [24]. Briefly, collected cells were lysed immediately in buffer [1% Triton X-100, 150 mM NaCl, 25 mM Tris–HCl (PH 7.2), 0.5 mM EDTA, 0.5 μM Na_3VO_4] supplemented with a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Protein concentration was determined using Micro BCA kit (Beyotime Biotechnology, Haimen, China). Equal amounts of protein (60 μg) were boiled for 5 min, separated by SDS–PAGE, and electroblotted to nitrocellulose membrane. After blocking, the blots were incubated with an appropriate dilution of specific antisera or monoclonal antibodies (Mcl-1, Bcl-2, Bcl-xL, Bax, Bak, Bik, Bid, XIAP, procaspase-9, -3, -7, -8, PARP, Cell Signaling Technology, Beverly, MA, USA; c-IAP1 and c-IAP2, R&D, Minneapolis, MN,USA) for 1 h at room temperature. Blots were washed three times and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Blots were again washed three times and then developed using a chemiluminescence assay. Blots were stripped and reprobed for β-actin (Cell Signaling Technology, Beverly, MA, USA) to be used as a loading control.

2.8. RT–PCR

After drug treatment, RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and cDNAs were prepared after running reverse transcription reactions using 2 μg of total RNA. PCR was then performed using primers as follows: Mcl-1 [25]: forward primer 5'-CGGTAATCGGA CTCAACCTC-3', and reverse primer 5'-CCTCCTTCTCCGTAGC CAA-3'; β-actin [26]: forward primer 5'-ACACTGTGCCCA TCTACGAGG-3', and reverse primer 5'-AGGGGCCGGACTCG TCATACT-3'. The reaction for Mcl-1 and β-actin was run for 30 and 25 cycles respectively. PCR products were separated on a 1.5% agarose and visualized under UV light by ethidium bromide staining.

3. Results

3.1. Lycorine inhibits the growth of leukemia cell lines

Fig. 2A shows the results of treating human leukemia cell lines K562, U937, HL-60, 6T-CEM and K562/G01 with increasing concentrations of lycorine. After 72-h treatments, lycorine decreased the survival of all five cell lines in a dose-dependent manner, with IC_{50} values for different cell lines ranging from 1.5 to 5.5 μM. Fig. 2B demonstrates that the effects of lycorine were time-dependent. In contrast to the leukemia cell lines, the viability of the normal PBMNCs was not significantly influenced by the same treatment at lycorine concentrations of up to 50 μM (Fig. 2C).

3.2. Lycorine induces apoptosis in leukemia cell lines

As shown in Fig. 2D, the treatment of 10 μM lycorine induced remarkable DNA fragmentation in K562, U937, HL-60 and 6T-CEM cells. Fig. 2E shows that the apoptotic populations increased in a dose-dependent manner in K562 or 6T-CEM cells exposed to lycorine for 24 h. These results strongly support that lycorine decreases survival of human leukemia cells through the induction of apoptosis.

3.3. Lycorine induces apoptosis through cytochrome C release and caspase activation

Fig. 3A shows that in K562 cells treated with lycorine for 24 h, cytochrome *C* was released from mitochondria to cytosol and procaspase-9 was cleaved into P37/35 in a lycorine dose-dependent manner. In these cells, cleaved products of procaspase-3, procaspase-7 and their substrate PARP were also detected. However, no discernible cleaved products of procaspase-8 could be measured. Analogous to the results in K562 cells, a lycorine dose-dependent cleavage of procaspase-3 and PARP was observed in HL-60 and 6T-CEM cells (Fig. 3B).

To determine whether the activation of the caspase cascade is necessary for lycorine-induced apoptosis, we examined the apoptosis inducing effects of lycorine in the presence of a pancaspase inhibitor Boc-D-fmk. K562 cells were pretreated with 50 μM of Boc-D-fmk for 1.5 h, and then incubated with 10 μM of lycorine for 16 h. As shown in Fig. 3C, Boc-D-fmk significantly decreased the apoptotic population induced by lycorine. This indicates that caspase activation is indeed required.

3.4. Lycorine triggers a rapid-turnover of protein levels of Mcl-1

Fig. 4A demonstrates that after 24 h of treatment by lycorine, the protein levels of Bcl-2 family proteins Mcl-1 and Bcl-xL declined in a dose-dependent manner in K562 cells. The same figure also suggests no discernible changes in the levels of Bax, Bak, Bik, Bid, XIAP, c-IAP1 and c-IAP2 after the treatments. Consistent with a previous report [27], the Bcl-2 protein itself was hardly detectable in the same cells.

Fig. 4B shows the time dependence of Mcl-1 and Bcl-xL protein levels and cleavages of caspase-3 and PARP in K562 cells incubated with 10 μM of lycorine for 1–12 h, The protein level of Mcl-1 started to decrease in as early as 1 h, whereas caspase-3 activation and PARP cleavage emerged in 2 h. The down-regulation of Bcl-xL was only observed in 12 h after lycorine administration. Previous reports had indicated that Mcl-1 protein can be cleaved by activated caspase-3 [28]. To confirm our results showing the earlier declining of the Mcl-1 level than caspase-3 activation, we treated K562 cells with 10 μM of lycorine for 6 h in both the presence and absence of the pancaspase inhibitor Boc-D-fmk. As shown in Fig. 4C, Boc-D-fmk could completely block the activation of caspase-3, but it was ineffective in preventing down-regulation of Mcl-1. Furthermore, Fig. 4D shows that Mcl-1 levels in K562 and HL-60 cells were higher than that in U937 and 6T-CEM cells and the former cells are associated with lower IC_{50} value for lycorine (Fig. 2A). Collectively, these findings suggest that a rapid-turnover of protein levels of Mcl-1 may mediate lycorine-induced apoptosis in leukemia cells.

3.5. Lycorine decreases Mcl-1 protein level at the post-transcriptional level

Fig. 5A shows that after 24 h of exposure to 10 μM of lycorine, there was no detectable change in the mcl-1 mRNA level in any of the three cell lines checked by RT-PCR. These results suggest lycorine reduces the Mcl-1 protein level through a mechanism other than transcriptional regulation. Fig. 5B demonstrates that the proteasome degradation system inhibitor MG-132 could impede the decrease of Mcl-1 induced by lycorine, and the treatment with MG-132 alone for 8 h led to an increase of Mcl-1 protein level. Interestingly, MG-132 did not decrease lycorine-induced apoptotic population detected by FACS assay. Conversely, more apoptotic population was observed after combined treatments (Fig. 5C). Thus lycorine also seems not to interfere with the proteasome degradation system.

3.6. Lycorine induces apoptosis in primary human leukemia blasts

To further understand whether lycorine can also induce apoptosis in primary human leukemia cells, blasts isolated from one patient (FAB classification M2) were incubated with lycorine for 24 h. As shown in Fig. 6A, treatment with 10 μM of lycorine resulted in 50.3% apoptosis in a sample from one patient. PARP protein was cleaved in a dose-dependent manner.

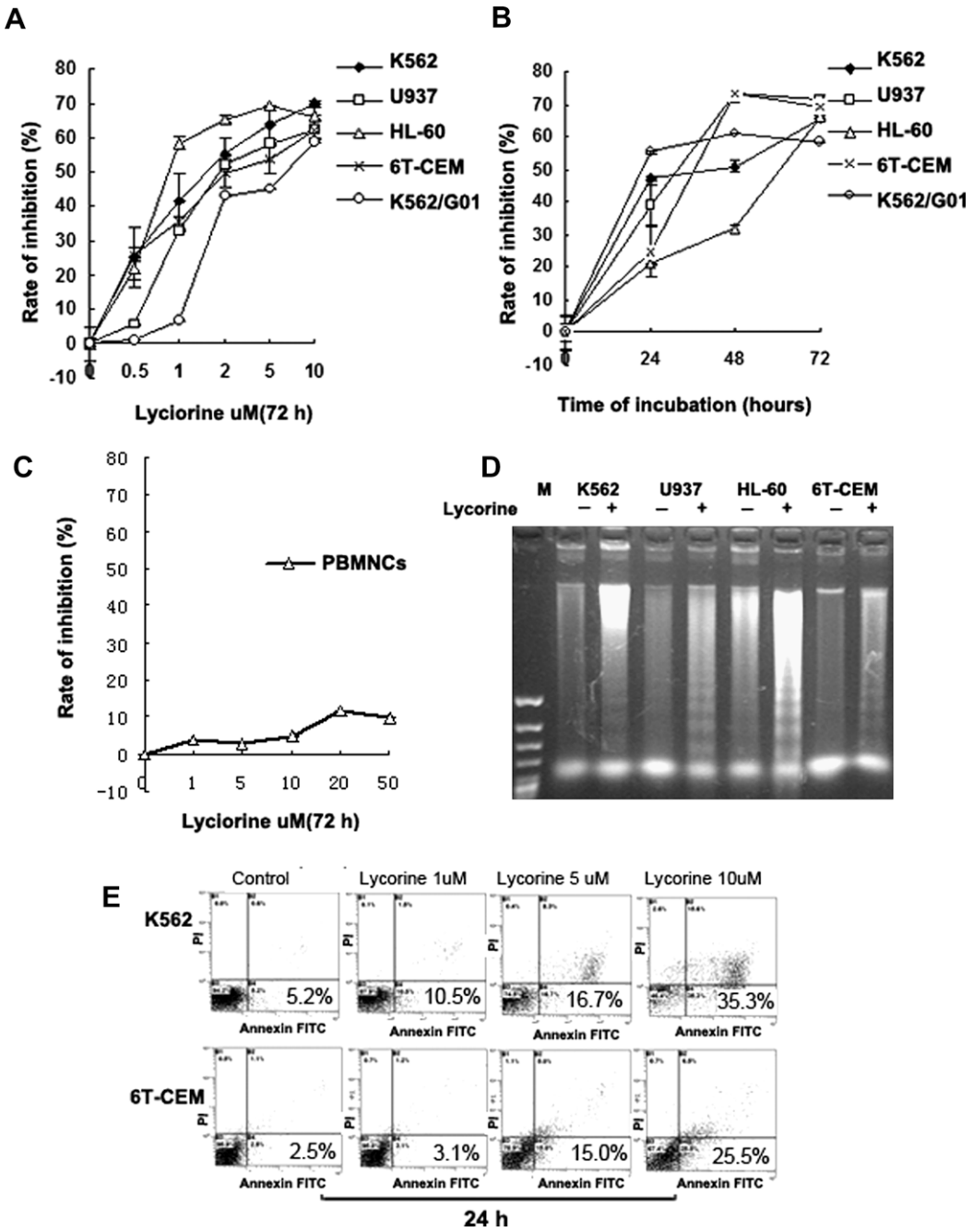

Fig. 2. Lycorine induces cytotoxicity and apoptosis in leukemia cell lines. (A) Cell viability in a dose-dependent manner was determined by MTT assay after 72-h culture. (B) Cell viability in a time-dependent manner was determined by MTT assay after incubation with 10 μM of lycorine. (C) Viability of PBMNCs derived from five healthy subjects was assessed by MTT assay after 72-h culture. (D) DNA fragmentation was detected after 24 h of exposure to 10 μM of lycorine. (E) The extent of apoptosis was assessed using Annexin V assay after 24-h incubation with the indicated concentrations. Data of MTT assay represent mean plus or minus the standard deviation (SD) of three independent experiments. Each experiment was performed two times and similar results were obtained.

Notably, a remarkable decrease in Mcl-1 protein level was also observed (Fig. 6B). Similar increase of apoptotic population induced by lycroine was also obtained in blasts from other two patients (Fig. 6A).

4. Discussion

Lycorine is a natural product with selective antitumor activities. More recently, this agent was reported to suppress the growth of human myeloid leukemia HL-60 cells both in vitro and in vivo [19,21]. In the present study, we investigated the effects of lycorine on the cell growth and apoptosis of a panel of human leukemia cell lines. We found that lycorine inhibited the growth or survival not only of HL-60 cells but also of other leukemia cells including the imatinib-resistant K562/G01 cell line. Although lycorine has been found to inhibit tumor cell apoptosis

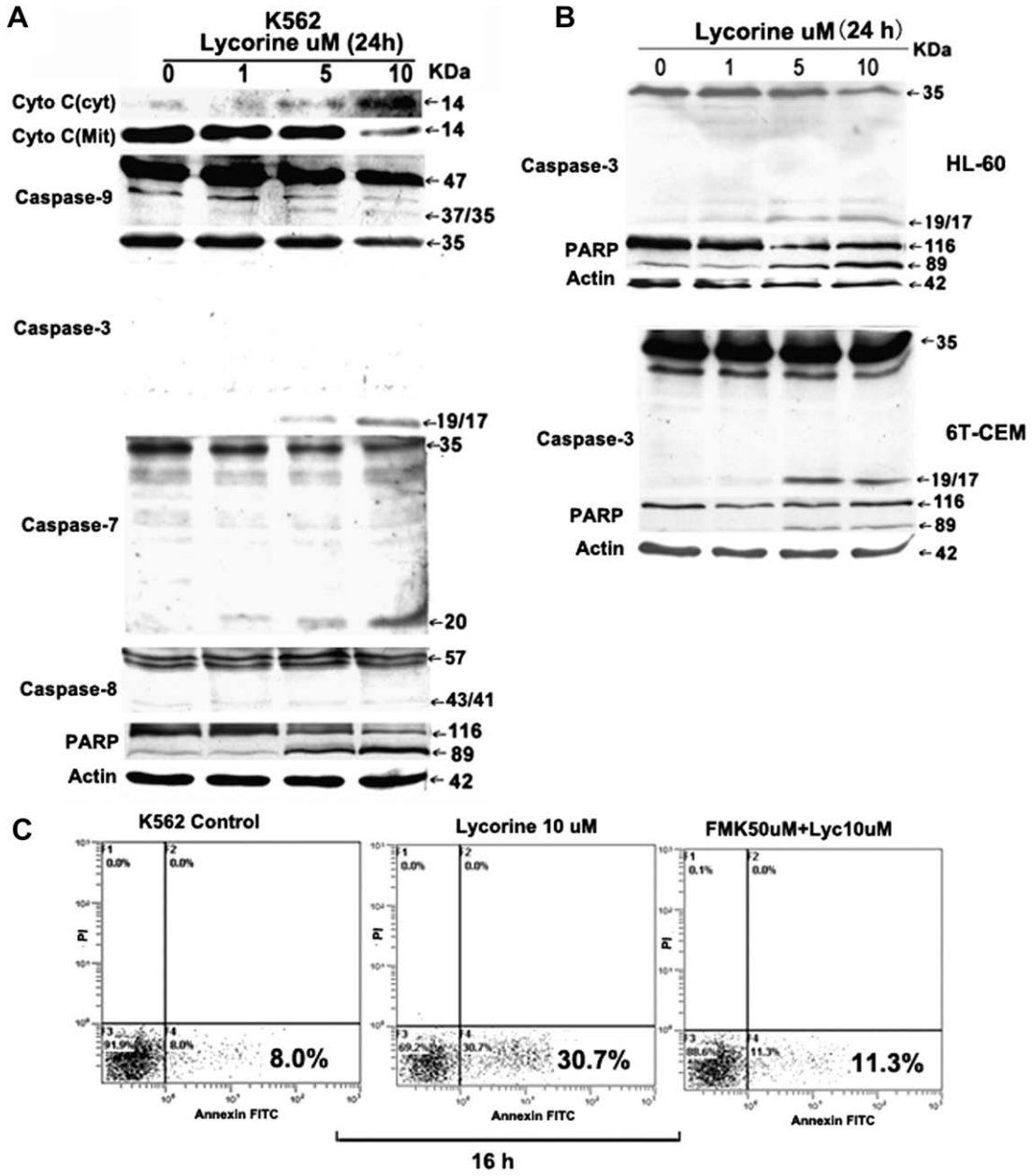

Fig. 3. Treatment with lycorine increases cytochrome *C* release from mitochondria and caspases activation. (A) After K562 cells were exposed to the indicated doses of lycorine for 24 h, both mitochondria and mitochondria-free cytosolic fractions were obtained and subjected to Western blot analysis to monitor release of cytochrome *C*. Whole cell lysates were subjected to Western blot analysis of cleavage of caspases and PARP. (B) Cells had been treated with the indicated doses of lycorine for 24 h, whole cell lysates were subjected to Western blot analysis of cleavage of caspase-3 and PARP. (C) K562 cells were pretreated with 50 μM of Boc-D-fmk for 1.5 h, and then incubated for 16 h with 10 μM of lycorine, the extent of apoptosis was assessed using Annexin V assay. Each experiment was performed two times and similar results were obtained.

induced by polymorphonuclear leukocyte-derived calprotectin [29], we have demonstrated that lycorine reduces the survival of human leukemia cells through the induction of apoptosis.

In addition, we provide evidence that lycorine induced apoptosis in human leukemia cells through the cytochrome *C*-mediated, caspase-dependent pathway. Caspases, a family of cysteine proteases, have been identified as the major components of apoptosis. The molecular mechanisms underlining apoptosis involves two principle pathways to activate the caspase cascades. One is triggered by the activation of death receptors on the cell surface by extracellular signals, and the other is related to the release of cytochrome *C* from mitochondria by intracellular signals. The two pathways subsequently transmit the signal to the downstream caspases including caspase-3 and degrade the DNA repair machinery associated with nuclear enzyme poly (ADP-ribose) polymerase (PARP) and other

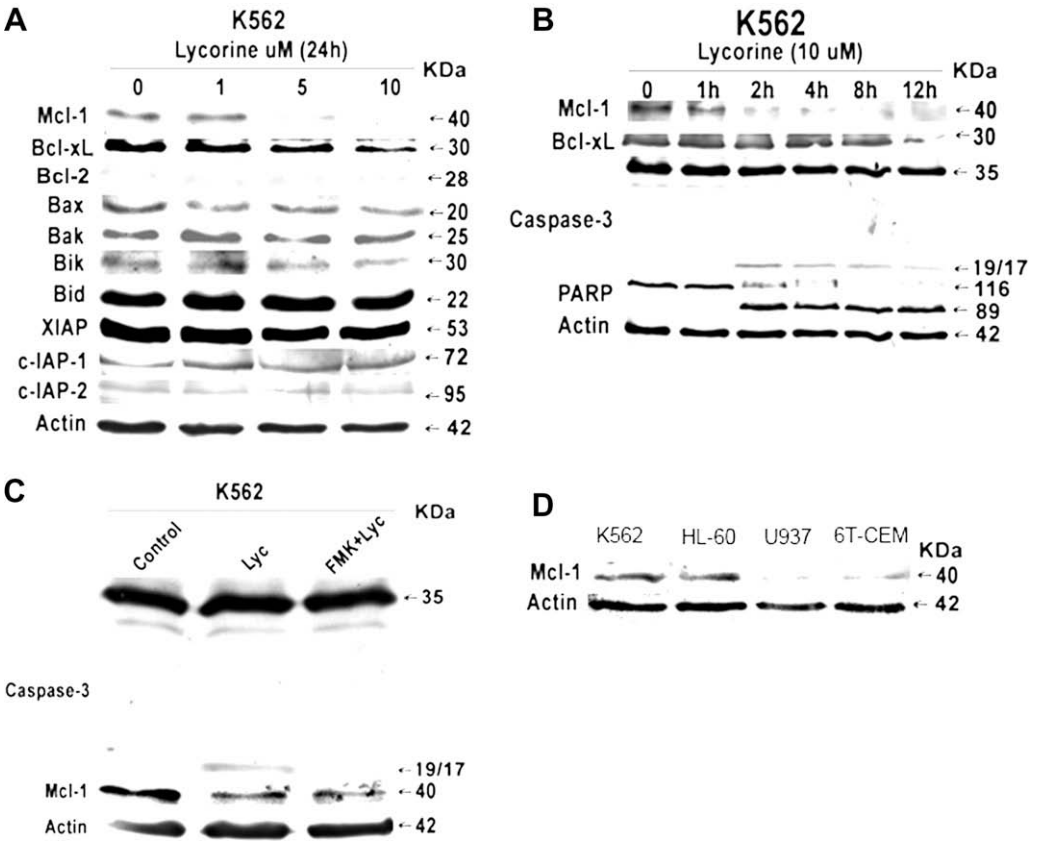

Fig. 4. Treatment with lycorine results in down-regulation of protein levels of Mcl-1. (A) Western blot analysis was conducted to assess the expression of bcl-2 family members and antiapoptotic proteins after K562 cells in exposure to the indicated doses of lycorine for 24 h. (B) K562 cells were treated with 10 μM of lycorine for the indicated time, after which Western blot analysis was performed using the indicated antibodies. (C) K562 cells were treated with 10 μM of lycorine for 8 h, with or without preincubation 50 μM of Boc-D-fmk for 1.5 h, after which cell lysates were subjected to Western blot analysis to assess Mcl-1 protein levels and activation of caspase-3. (D) Western blot analysis was conducted to assess protein levels of Mcl-1 in distinct cell lines. Each experiment was performed two times and similar results were obtained.

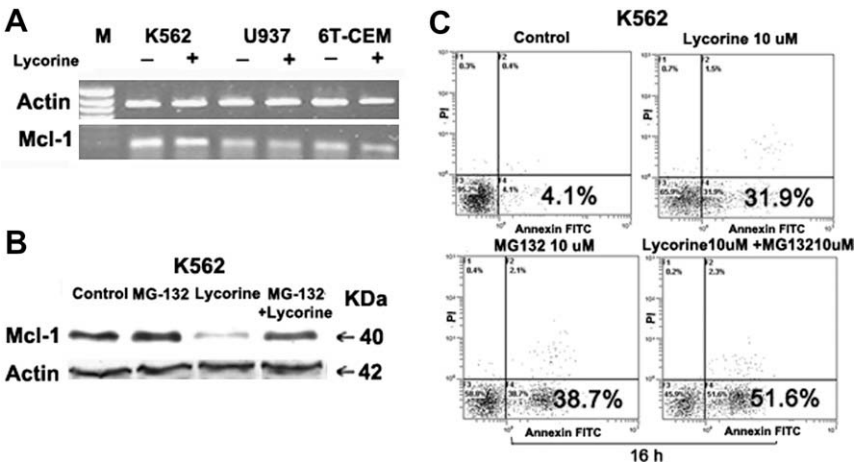

Fig. 5. Lycorine treatment decreases Mcl-1 protein levels at the post-transcriptional level. (A) RT-PCR analysis of Mcl-1 and β-actin was performed after the indicated cell lines were treated with 10 μM of lycorine for 24 h. M means DAN size marker. (B) Western blot analysis was conducted to monitor Mcl-1 protein levels after K562 cells were incubated with 10 μM of lycorine in the absence or presence of 1 μM of MG-132 for 8 h. (C) The extent of apoptosis was assessed using Annexin V assay after K562 cells were incubated with 10 μM of lycorine in the absence or presence of 1 μM of MG-132 for 16 h. Each experiment was performed two times and similar results were obtained.

proteins [30]. In this study, we found that in leukemia cells, lycorine triggered a rapid release of cytochrome *C* from mitochondria to cytosol, leading to the activation of caspase-9 and its downstream caspases including caspases-

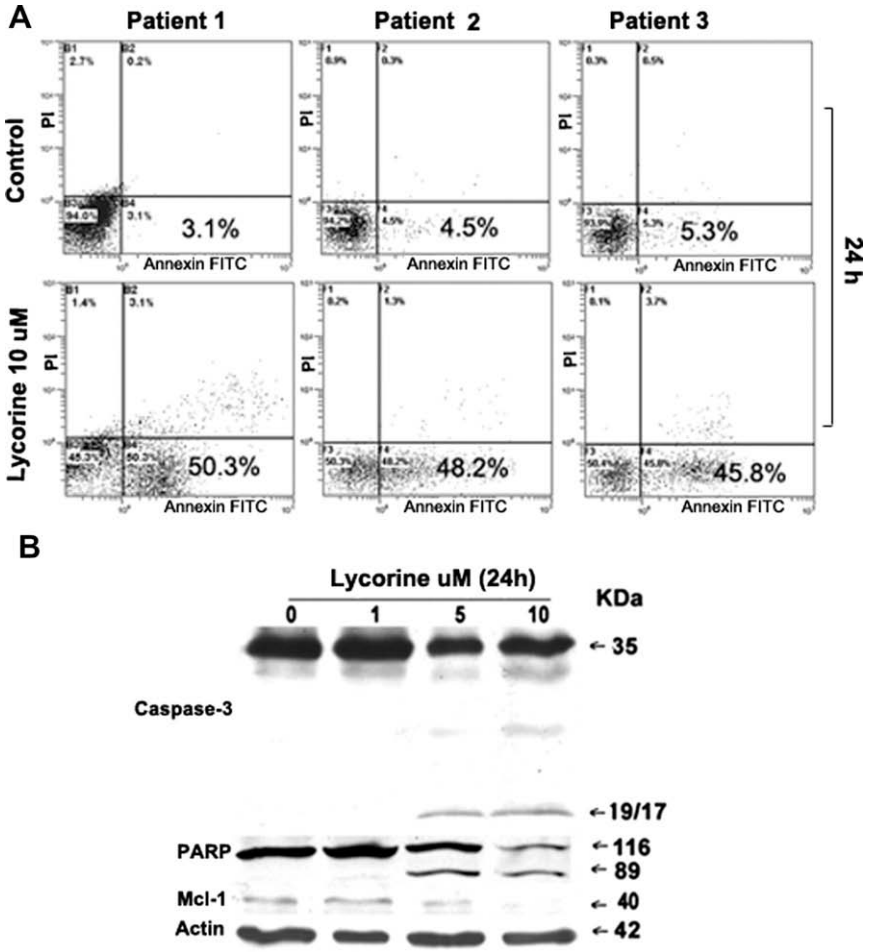

Fig. 6. Exposure to lycorine causes a remarkable increase of apoptosis in primary human leukemia blasts. After primary blasts were treated with 10 μM of lycorine for 24 h. (A) The extent of apoptosis was assessed using flow cytometry; (B) whole cell protein lysates were subjected to Western blot analysis of the indicated antibodies.

3, and -7, followed by the cleavage of their substrates including PARP. But no discernible cleaved products of procaspase-8 were measured. Consistently, we found that the pancaspase inhibitor Boc-D-fmk could suppress lycorine-induced apoptosis.

Our results also suggest that lycroine-induced leukemia cell apoptosis may be triggered through the reduction of a bcl-2 family protein, Mcl-1. Intensive studies on apoptosis induction by a variety of stimuli have indicated that the bcl-2 family members participate in the control of apoptosis by regulation of cytochrome *C* release from mitochondria [31]. According to a previous report, lycorine treatment seemed to change the protein levels of Bcl-2 and Bax in HL-60 cells [19]. However, the results obtained in our study were that lycorine induced apoptosis in K562 cells lacking Bcl-2 protein [27], with both protein levels of Bcl-xL and Mcl-l down-regulated in response to lycorine in a dose-dependent manner, and with Bax expression unaltered. We further observed that lycorine triggered a rapid Mcl-1 turnover at 1 h before the activation of the caspases, whereas protein level of Bcl-xL was not down-regulated until 12 h after caspase activation. The pancaspase inhibitor Boc-D-fmk could not prevent Mcl-1 down-regulation. The results suggest that Mcl-1 down-regulation is not a consequence of caspases activation and it may be the early event contributing to lycorine-induced apoptosis. Lycorine-induced apoptosis and Mcl-1 down-regulation were also observed in patient cell samples.

Mcl-1 is a short-lived protein with a half-life between 30 min and a few hours depending on the cell types and culture conditions [32,33]. Mcl-1 protein levels are known to be regulated through several mechanisms, including those operating at the transcriptional, translational, and post-translational levels [25,34–37]. More recently, reports have also shown that translation inhibition by protein inhibitors, such as semisynthetic homoharringtonine (ssHHT), one compound with clinical effects of antileukemia [38], and cycloheximide, a global inhibitor of translation [39], leads to Mcl-1 turnover, and this plays a critical role in apoptosis induced by these agents. Lycorine is a natural compound with a variety of biological functions. Although the molecular mechanisms underling the effects of lycorine on eukaryotic cells remain unclear, lycorine has

been reported to inhibit protein synthesis [18,40]. The present results reveal that lycorine induced a rapid and sharp decline in Mcl-1 protein levels. Although proteasome inhibitor MG-132 blocked the lycorine-induced decrease of Mcl-1, combination treatment led to more apoptotic cells. These results supports the idea that lycorine may induce apoptosis with down-regulation of Mcl-1 at translational level rather than at degradation level.

Mcl-1 plays a critical role in survival of malignant hematopoietic cells and the development of compounds that diminish Mcl-1 protein levels has been the focus of intense interest. Here we have demonstrated that lycorine exerts strongly cytotoxicity against leukemia cells by inducing apoptosis, probably through translational inhibition of the Mcl-1 protein which leads to a rapid reduction of the amount of this protein in leukemia cells. This phenomenon has been observed not only in cultured cell lines, but also in patient samples. In addition, we also found that lycorine treatment efficaciously suppressed the growth of imatinib-resistant K562/G01 cells. Our results suggest that it is worth further studying to determine whether lycorine is a good candidate therapeutic agent against hematological malignancies, especially relapse leukemia in which Mcl-1 accumulation causes resistance to therapy.

Conflict of interest statement

None declared.

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