

# Apoptosis induced by lycorine in KM3 cells is associated with the G0/G1 cell cycle arrest

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**Abstract.** Lycorine is a natural anti-tumor alkaloid extracted from Amaryllidaceae and has various biological effects on malignant cells. The present study explores the effects of lycorine on the human multiple myeloma cell line, KM3, and the possible mechanisms of these effects. An MTT assay showed that lycorine had significant inhibitory activity on KM3 cells. The growth rates of the KM3 cells exposed to lycorine evidently slowed down. Cell fluorescent apoptotic morphological changes, DNA degradation fragments, and a sub-G1 peak were detected, indicating the occurrence of cell apoptosis after lycorine treatment. Furthermore, the release of mitochondrial cytochrome c, the augmentation of Bax with the attenuation of Bcl-2, and the activation of caspase-9, -8, and -3 were also detected, suggesting that the mitochondrial pathway and the death receptor pathway were also involved. The results also showed that lycorine was able to block the cell cycle at the G0/G1 phase through the downregulation of both cyclin D1 and CDK4. In summary, lycorine can suppress the proliferation of KM3 cells and reduce cell survival by arresting cell cycle progression as well as inducing cell apoptosis.

## Introduction

Multiple myeloma (MM), a hematological disorder, is characterized by the malignant proliferation of monoclonal plasma cells in bone marrow. Currently, MM represents over 10% of the malignant tumors in hematological systems around the world. It is the second most frequent hematological malignancy, with a 5-year survival rate of <20% (1,2). With

the aging of the population, the incidence of MM inevitably rises. Therefore, the perniciousness of MM is still a serious issue in the world (3).

Plasma cells undergo a multistep transformation process before myeloma is formed. The presence of somatic hypermutations of immunoglobulin variable-region genes is consistent with an immortalizing event during plasma cell generation in the germinal centers of the lymph nodes (4,5). Genomic instability is already present in monoclonal gammopathy of undetermined significance (MGUS) (6,7). MGUS cannot be distinguished from myeloma at the gene-expression level, although the plasma cells of both MGUS and myeloma can be clearly distinguished from healthy bone marrow plasma cells (8). Evidence demonstrating that myeloma cells have short telomeres and that healthy plasma cells have long telomeres, could be applied to MGUS, in order to help distinguish subsets with different propensities for progression to overt disease (9).

The failure of myeloma cells to undergo apoptosis plays an important role in their accumulation within the bone marrow (10). The Bcl-2 family proteins, consisting of anti-apoptotic and pro-apoptotic members, determine the life or death of a cell (11). Bcl-2 is a highly conserved, ubiquitous membrane protein associated most often with the outer membranes of mitochondria and of nuclei and with the endoplasmic reticulum that regulates apoptosis (12). Bcl-2 is a survival factor for many cell types, and the overexpression of Bcl-2 in cancer cells can result in chemoresistance (13). Bcl-2 plays a major role in drug resistance both in myeloma cell lines and in freshly isolated myeloma cells. Furthermore, apoptosis induced by dexamethasone (14-16), paclitaxel (16), and adenovirus-mediated p53 (Ad-p53) is blocked in the cells expressing high levels of Bcl-2 and in Bcl-2-transfected cell lines (17,18).

Investigations into the molecular mechanisms governing the G1 to S phase progression in mammalian cells demonstrated a central role for D-type cyclins and for cyclin-dependent kinases (CDKs) 4 and 6 (19,20). The cell-cycle-accelerating function of cyclin D-associated kinases is mediated by the phosphorylation and concomitant inactivation of the retinoblastoma protein (pRb) in the G1 phase, a process negatively regulated by p16<sup>INK4A</sup> and other CDK inhibitors. The emerging critical role of the cyclin D1, pRb, and p16<sup>INK4A</sup> pathway in cell cycle regulation is further supported by the frequent alterations of the individual components of this checkpoint

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mechanism in human tumors (21,22). pRb, p16<sup>INK4A</sup>, cyclin D1, and the CDKs 4 and 6 constitute a G1 regulatory pathway commonly targeted by myeloma tumorigenesis (23).

Lycorine, an alkaloid extracted from amaryllis plants, has been shown to exhibit various natural anti-tumor activities. Lycorine can halt protein synthesis in eukaryotic cells by inhibiting peptide bond formation (24), suppress viral protein synthesis in poliovirus-infected Hela cells (25), stop the acetylcholinesterase enzyme action (26,27), and exhibit anti-malarial activity (28). Alternatively, lycorine can inhibit the protein synthesis and cell apoptosis of MM46 in the presence of calprotectin (29). Lycorine can suppress leukemia cell growth and reduce cell survival by arresting the cell cycle and inducing the apoptosis of tumor cells (30).

The human MM cell line, KM3, is one of the pre-B lymphoid cell lines having such differentiation capabilities and is an eternal cell line derived from the bone marrow of clinical MM patients (31). In the present study, we used the KM3 cell line as a model to observe the effect of lycorine on MM cells and to explore its possible molecular mechanisms. Our data demonstrate that lycorine can use different pathways to induce apoptosis and cell cycle arrest in KM3 cells.

## Materials and methods

**Cell lines and chemicals.** The human MM cell line KM3 was supplied by Dr J. Hou (Department of Hematology, Changzheng Hospital, The Second Military Medical University, Shanghai, P.R. China). The human embryonic kidney epithelial cell line, HEK293, and the fibroblast cell line, NIH/3T3, were from the American Type Culture Collection (ATCC, USA). The KM3, NIH3T3, and HEK293 cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal calf serum. All culture media contained 100 U/ml penicillin, 100 µg/ml streptomycin, and 4 µg/ml amphotericin B. The cell lines were carefully cultured in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Lycorine (Latoxan, France) was dissolved at 0.03 M in dimethyl sulfoxide (DMSO; Sigma, USA) as a stock solution and then diluted in serum-free RPMI-1640 medium just before use. Melphalan was purchased from Sigma.

**Cell survival rate and cytotoxicity assay (MTT).** The cell survival rate and cytotoxicity were measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay, with slight modifications. Briefly, exponentially growing KM3, NIH3T3, and HEK293 cells were seeded at 1×10<sup>5</sup> cells/well in a 96-well tissue culture plate (BioCoat, USA) with a total volume of 200 µl per well. Lycorine concentrations ranging from low to high concentrations (5 assay points) were added immediately. The same process was used for melphalan. After the cells were incubated for 48 h, the relative cell viability was measured by scanning with an ELISA reader using a 570-nm filter, and we calculated the 50% inhibitory concentration (IC<sub>50</sub>) of lycorine on the cells. Values were expressed as the percentages of the drug-treated cells relative to those of the untreated cells (100%).

**Inhibition of growth curve.** Exponentially growing KM3 cells were seeded at 0.5×10<sup>5</sup> cells/well in a 12-well tissue culture

plate (BioCoat) with a total volume of 1000 µl per well. A final concentration of 1.25 µM lycorine was added immediately to 6 parallel wells. Another 6 wells were used as the controls. After incubation for each 24 h (5 assay points), the cells were immediately counted using the trypan blue dye exclusion method, and a cell growth curve was drawn.

**Detection of apoptotic cells by fluorescence staining.** The apoptotic morphology of the cells exposed to the indicated concentrations of lycorine for 24 h was monitored by Hoechst33258 (Sigma) staining and then observed with fluorescent microscopy, as previously described (32). For each experiment, the cells were stained in a culture medium containing 1 mg/ml of the dye, and at least 500 cells with condensed/fragmented nuclei were scored to determine apoptosis.

**Determination of DNA fragmentation.** More than 1×10<sup>7</sup> KM3 cells were harvested which had been incubated for 48 h either with different lycorine concentrations or without lycorine. Total DNA was extracted according to the procedure used by Sellins and Cohen (33). DNA fragmentation was analyzed on 2% agarose gels, and then visualized under UV light by ethidium bromide staining.

**Analysis of apoptotic cells by flow cytometry.** As indicated, at different times or concentrations, the cells were harvested and washed 3x with phosphate-buffered saline (PBS). Then the cells were fixed with 70% cold alcohol at 4°C for 24 h. Before assessment, the cells were incubated with 100 µg/ml RNase A at 37°C for 30 min and then stained with 50 µg/ml propidium iodide (PI; Sigma) at 4°C for 20 min, and kept away from light. The cell cycle was measured with FACS-caliber flow cytometry and analyzed by Cell Quest software.

**Release of cytochrome c from mitochondria.** KM3 cells, treated with lycorine for 24 h, were harvested, washed with cold PBS, and centrifuged. The cell pellets were smeared on a slide and fixed with 4% formaldehyde for 30 min. Non-specific antigens were blocked by 1:20 normal serum diluted with PBS for 10 min at room temperature. Then the cytochrome c in the cytoplasm was measured using the cytochrome c assay kit (BioVision, USA). The protein distribution was measured using a confocal laser microscope with an excitation light at a wavelength of 554 nm.

**Detection of caspase activity.** The caspase assay system (BioVision) was applied to determine caspase activities according to the manufacturer's instructions. The cleavage activities of IETD-pNA, LEHD-pNA, and DEVD-pNA were measured by using the FLICE/caspase-8, Mch6/caspase-9, and CPP32/caspase-3 colorimetric assay kit, respectively. Exponentially growing KM3 cells (5×10<sup>6</sup>) in 5 ml were treated with lycorine (5 µM) or without lycorine as control for 0, 6, 12, 18, and 24 h at 37°C. At the indicated time points, the cells were pelleted by centrifugation and resuspended in a 50 µl chilled cell lysis buffer. The protein concentration was measured using the BCA protein assay kit (Pierce, USA). Protein (100 µg) was diluted with 50 µl cell lysis buffer for each assay, and 50 µl 2x reaction buffer supplemented with

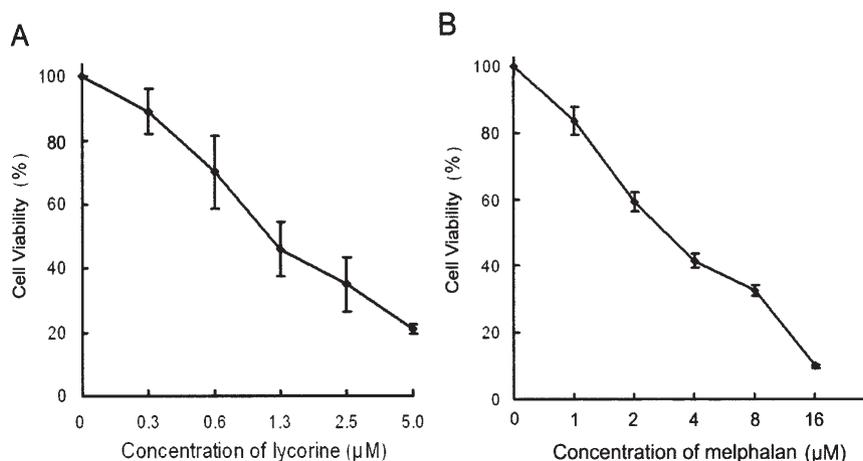


Figure 1. The effects of lycorine or melphalan on the cell survival rates of the human multiple myeloma cell line, KM3. The KM3 cells were cultivated in RPMI-1640 medium with indicated concentrations of lycorine or melphalan for 24 h. The cell survival rate was calculated by MTT assay. (A) The effects of lycorine on the cell survival rates of the KM3 cells. (B) The effects of melphalan on the cell survival rates of the KM3 cells. The values are expressed as the means  $\pm$  S.D. of 3 independent experiments.

10 mM DTT was then added to each tube incubated at 4°C. The substrates of IETD-pNA, LEHD-pNA, and DEVD-pNA were added into the tubes (5  $\mu$ l and 50  $\mu$ M), respectively, and then incubated at 37°C for 1.5 h. The formation of p-nitroanilide in the samples was measured with an ELISA Microplate Reader (EC-800, USA) at 405 nm. The values were expressed as the percentages of caspase-8, -9, -3 activities relative to those of the untreated cell extract (100%).

**Western immunoblot analysis.** Exponentially growing KM3 cells ( $2 \times 10^6$ ) treated with 0, 2.5 and 5  $\mu$ M lycorine for 24 h, were pelleted by centrifugation, washed 3x with 2 ml PBS, resuspended in 100  $\mu$ l lysis buffer containing 20 mM HEPES, pH 7.4; 100 mM NaCl; 1% Nonidet P-40; 2% sodium dodecyl sulfate (SDS); 1% deoxycholic acid; 10% glycerol; 1 mM EDTA; 1 mM EGTA; 1 mM sodium orthovanadate; 50 mM sodium fluoride; 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 mg/ml leupeptin; 1 mg/ml aprotinin, and sonicated. The protein concentration was measured using a micro BCA kit (Pierce). Protein (80  $\mu$ g) was electrophoresed for 2 h on 12%-SDS polyacrylamide gels, then transferred to polyvinylidene difluoride membranes (Millipore, USA) using a semi-dry electroblotter for 12 h at 4°C. Antibodies (Santa Cruz Biotechnology, USA) raised against Bcl-2, Bax, cyclin D1 (1:200), CDK4 (1:500), or  $\alpha$ -tubulin (1:4000), were diluted in phosphate-buffered saline Tween-20 (PBST) containing 5% non-fat milk in all the cases. The membranes were incubated for 2 h with gentle agitation. The blots were washed 3x (10 min each) with PBST and incubated with sheep anti-mouse conjugated to horseradish peroxidase (Santa Cruz Biotechnology; 1:2000 dilution in PBST containing 5% non-fat milk) for 2 h. After 3 successive washings with PBST, a chemiluminescence reagent (ECL) was used for signal detection. The optical density of the immunoradiograms was quantified by densitometric scanning.

**Statistical analysis.** The results were expressed as the means  $\pm$  SD. The statistical differences between the groups were

determined by the one-way analysis of variance (ANOVA) and Tukey's Studentized Range test. Differences among the groups were considered statistically significant at  $P < 0.05$ .

## Results

**Lycorine effectively decreased cell survival rate.** MTT assays showed the survival rate of the KM3 cells exposed to lycorine (0.3-5  $\mu$ M) for 48 h. This exposure was reduced in a dose-dependent manner, and thus the viability of the cells was decreased from  $89 \pm 6.5$  to  $21 \pm 1.4\%$  (Fig. 1A), with an  $IC_{50}$  value of 1.25  $\mu$ M. We also examined the effects of lycorine on 2 other non-tumor cell lines, the mouse fibroblast cell line, NIH3T3, and the human recombinant cell line, HEK293. Among the tested cell lines, KM3 proved to be the most sensitive to the inhibitory effects of lycorine, compared to the little sensitivity detected in the non-tumor cells, NIH3T3 and HEK293, which is indicative of the selective toxicity of lycorine (data not shown).

Melphalan, frequently used in the clinical treatment of MM, was tested as the positive control. After 48 h, the survival rate of the KM3 cells treated with 1 to 16  $\mu$ M melphalan at a half-effective concentration of 3.2  $\mu$ M, dropped from  $84.2 \pm 6.5\%$  to  $10.2 \pm 3.3\%$  (Fig. 1B). When comparing the  $IC_{50}$  values of lycorine and melphalan, it is obvious that the tumor cells were more sensitive to lycorine-induced cytotoxicity than to melphalan. The  $IC_{50}$  value of lycorine was  $\sim 1/2.5$  that of the melphalan value. Thus, the cellular toxicity of lycorine is 2.5x greater than that of melphalan. Therefore, lycorine demonstrates a stronger suppressive effect on MM cells.

**Cell proliferation inhibited by lycorine.** The KM3 cells were treated with 1.25  $\mu$ M lycorine and examined by cell counting every day for 5 days. Cell proliferation was significantly inhibited in a time-dependent manner. The density of the lycorine-treated KM3 cells decreased significantly to about one half the density of the control (Fig. 2).

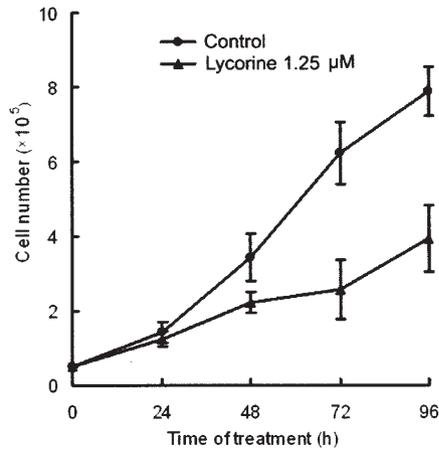


Figure 2. The effects of lycorine on the proliferation of the KM3 cells. The KM3 cells were treated by 1.25  $\mu\text{M}$  lycorine for the indicated time. The cell proliferation was estimated by the trypan blue dye exclusion method. The curve shows the growth and proliferation of the KM3 cells inhibited by 1.25  $\mu\text{M}$  lycorine in a time-dependent manner. The values are expressed as the means  $\pm$  S.D. of 3 independent experiments.



Figure 4. DNA fragmentation of the KM3 cells induced by lycorine. After lycorine treatment for 48 h, the cellular DNA was extracted and ran on 1.5% agarose gels, and subsequently visualized under UV light by ethidium bromide staining. Lane 1, without treatment as the control; lane 2, 2.5  $\mu\text{M}$  lycorine treatment; lane 3, 5  $\mu\text{M}$  lycorine treatment; lane 4, 10  $\mu\text{M}$  lycorine treatment.

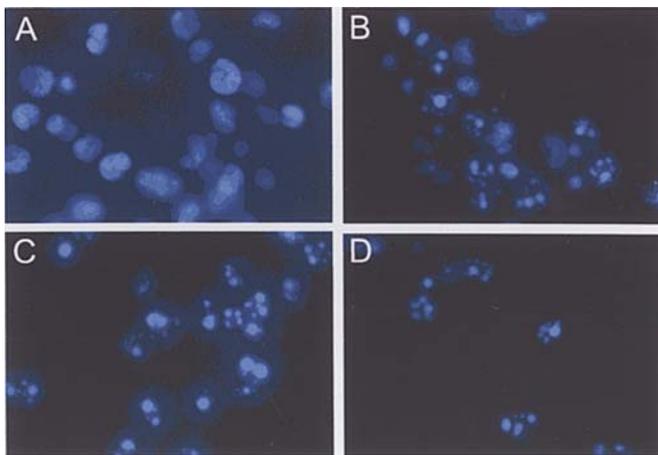


Figure 3. Lycorine-induced apoptosis of the KM3 cells by Hoechst33258 staining. The KM3 cells were cultured for 48 h with the indicated concentration of lycorine. (A) Cultures without drugs as the control (5.7% apoptosis). (B) Cultured with 2.5  $\mu\text{M}$  lycorine, the cells showed a light condensation of chromatin and nuclei margination (32.8% apoptosis). (C) Cultured with 5  $\mu\text{M}$  lycorine, the cells showed fragmented chromatin and apoptotic bodies (50.4% apoptosis). (D) Typical apoptotic bodies in most of the KM3 cells were seen after treatment with 10  $\mu\text{M}$  lycorine (66.1% apoptosis) (x400).

**Enhancement of apoptosis induced by lycorine.** We examined the efficacy of the lycorine-induced apoptosis of the KM3 cells by staining the cells with Hoechst33258 and observing them with a fluorescence microscope. All the cells and their nuclei were dyed blue. Normal morphology and nuclei were observed in the control group (Fig. 3A). The 2.5  $\mu\text{M}$  group (Fig. 3B) showed light nuclear margination, whereas typical apoptotic cells with fragmented chromatin or apoptotic bodies were seen after the 5  $\mu\text{M}$  and 10  $\mu\text{M}$  lycorine treatments (Fig. 3C and D). The apoptosis rate in the KM3 cells (including the cells in early apoptosis) was dose-dependent.

Agarose gel electrophoresis was used to observe DNA fragmentation, a typical step on the ladder during cell apoptosis. DNA fragmentation was observed when lycorine was applied at a concentration of 2.5  $\mu\text{M}$ . However, when a higher concentration of lycorine (5 or 10  $\mu\text{M}$ ) was applied, the DNA ladder was obvious (Fig. 4).

**Apoptotic rate change and cell cycle arrest.** We found that the apoptotic rates increased from 10.8 to 43.2% in a dose-dependent manner (0, 2.5, 5 and 10  $\mu\text{M}$ ) after 48 h of processing (Fig. 5A), and from 11.1% to 45.4% in a time-dependent manner (0, 24, 48 and 72 h) after treatment with 5  $\mu\text{M}$  lycorine (Fig. 5B). The flow cytometry assay also showed an increase in the population of cells at the G0/G1 phase, ranging from 28.6 to 41.4% in a time-response manner, after the KM3 cells were treated with 1.25  $\mu\text{M}$  lycorine (Fig. 5C), and from 25.4 to 62.5% in a dose-response manner after the application of different concentrations of lycorine for 48 h (Fig. 5D), demonstrating that the cell cycle was being arrested at the G0/G1 phase after the lycorine treatment.

**Influences in activation of caspase-8, -9, and -3.** A remarkable activation of caspase-8 and -3 in the lysates from the KM3 cells treated with 5  $\mu\text{M}$  lycorine was recorded at the indicated time points (Fig. 6). We could see that the high points of caspase-8 and -3 were at the 24-h elapsed treatment time, whereas the activation of caspase-9 increased slightly. The activation of caspase-3 increased the most significantly and the most quickly, representing a crucial factor in the caspase-cascade downstream pathway (34).

**Release of cytochrome c from mitochondria.** We examined the subcellular localization of cytochrome c to determine whether the cytochrome c was released from the mitochondria to the cytosol in the apoptosis pathway initiated by lycorine.

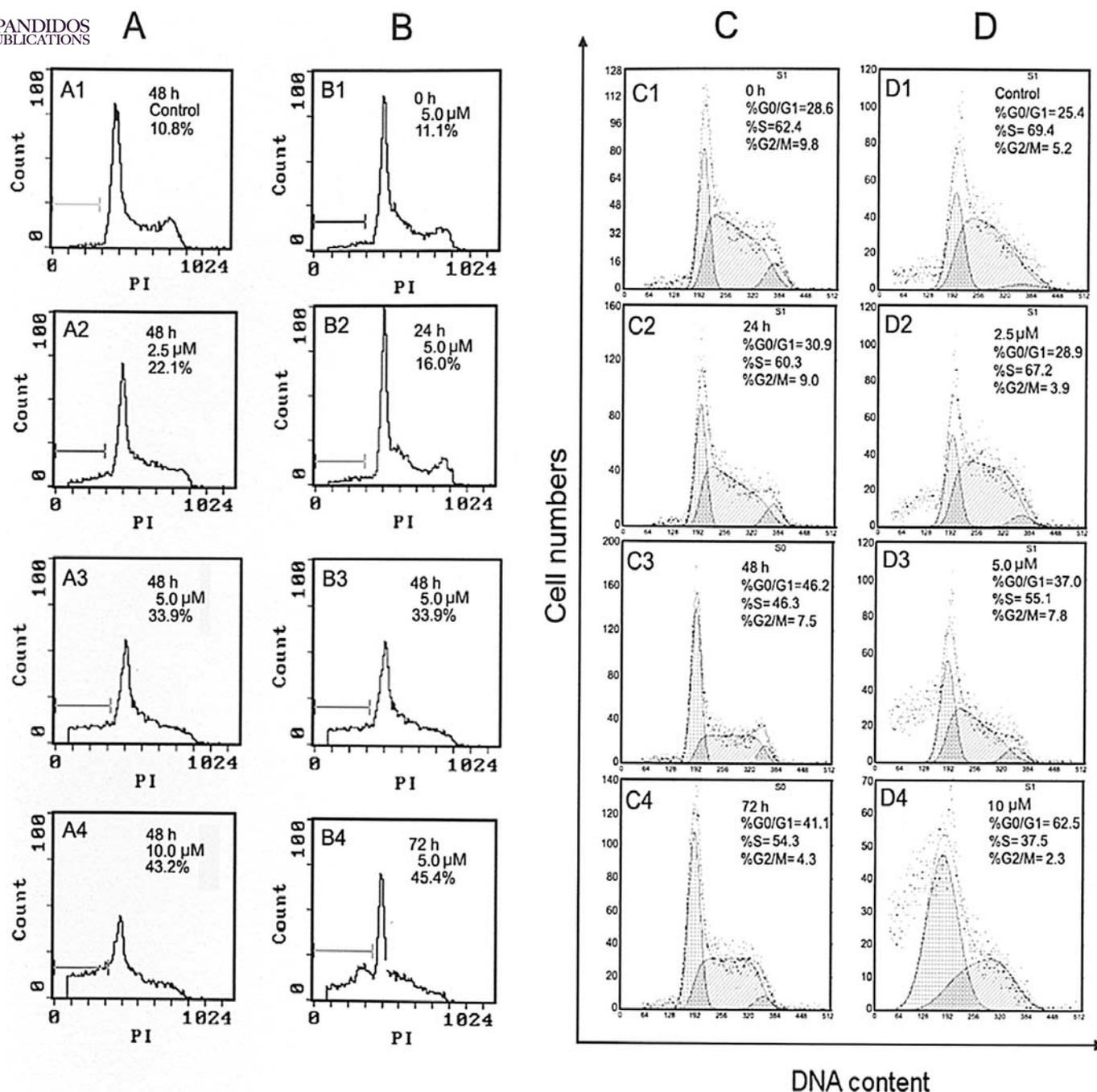


Figure 5. Lycorine augmented the apoptotic rates of the KM3 cells and the proportion of the G0/G1 phase. (A) The ratio of apoptosis increased from 10.8 to 43.2% after treatment with indicated concentrations of lycorine for 48 h. (B) The apoptotic percentage increased from 11.1 to 45.4% after 5  $\mu$ M lycorine treatment for indicated times. (C) The number of cells in the G0/G1 phase increased from 28.6 to 41.4% when the cells were treated with 1.25  $\mu$ M lycorine at different times. (D) The number of cells in the G0/G1 phase increased from 25.4 to 62.5% after treatment with different concentrations of lycorine for 48 h.

The immunofluorescence of cytochrome c was visualized under a confocal laser microscope. In both groups, the nucleus was observed to be blue, from staining the DNA with Hoechst33258 (Fig. 7A1 and B1) and the cytochrome c red, from staining with Cy3 fluorochrome (Fig. 7A2 and B2). After the cells were treated with 5  $\mu$ M lycorine for 24 h, the staining patterns of cytochrome c became diffuse and blurred (Fig. 7B2), contrasting with the compact-plaque appearance of cytochrome c in the control group (Fig. 7A2), indicating the translocation of cytochrome c from the mitochondria into the cytosol. We also found that some red dye entered the

nucleus, mixing with the blue stain to yield the purple staining of the nucleus (Fig. 7B3).

*Decreases in Bcl-2, cyclin D1, and CDK4 and an increase in Bax.* The effect of lycorine on the expression of Bcl-2, Bax, cyclin D1, and CDK4 proteins was determined by Western immunoblot analysis in order to explore the possible mechanisms underlying the lycorine-induced apoptosis of the KM3 cells (Fig. 8A).  $\alpha$ -tubulin was used to normalize and verify the protein loading. After being induced by lycorine, the Bcl-2, cyclin D, and CDK4 expression significantly

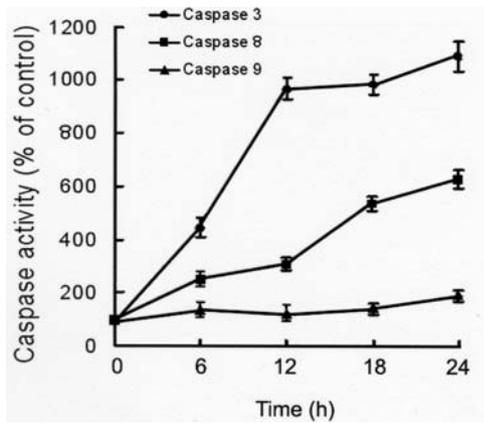


Figure 6. Determination of the activation of caspase-8, -9 and -3 in the KM3 cells treated with lycorine. The KM3 cells were treated with lycorine (5  $\mu$ M) for the indicated time before the caspase-8 substrate, IETD-pNA, the caspase-9 substrate, LEHD-pNA, or the caspase-3 substrate, DEVD-pNA (50 mM) were added. The mixtures were incubated for 1.5 h at 37°C followed by the measurement of the absorbance at 405 nm. The percentage increases in the caspase activities were determined by comparing the results at the indicated time points with the level of the control. Each value represents the means  $\pm$  S.D. of 3 independent experiments.

decreased in a dose-dependent manner, whereas the Bax protein levels increased slightly (Fig. 8B and C).

## Discussion

The knowledge about the importance of overcoming anti-apoptosis or directly inducing apoptosis in the treatment of a variety of malignancies, including MM, raises new hope of improving treatment outcomes for patients with cancer (35-37). The value of targeting therapies that aim to induce apoptotic processes in MM cells has been explored in a number of experimental systems, and many potential new therapies for leukemia are emerging, including using thalidomide (38), proteasome inhibitors (39), and arsenic compounds (40), although the use of these agents is still limited.

Our data show that lycorine significantly inhibited the proliferation of the human MM cell line, KM3, after treatment at a half-effective concentration of 1.25  $\mu$ M. Laser confocal microscopy and DNA fragmentation assay suggested the occurrence of apoptosis. The efficacy of lycorine in inducing MM cell apoptosis was proven in the present experiments.

Apoptosis plays critical roles in tissue homeostasis (41). Apoptosis is caused by the activation of the intracellular proteases known as caspases. When cleaved, numerous cellular targets of caspases can aggregate to produce the characteristic morphological changes called apoptosis. Several caspases, mediated by various anti-tumor drugs, have been shown to be key executors of apoptosis (42). Several pathways exist for triggering caspase activation, and between these, intrinsic and extrinsic pathways have been elucidated in detail and have been the center of attention in past years (43).

The intrinsic apoptotic pathway focuses on the mitochondria as the initiators of cell death. Multiple signals converge on mitochondria, causing these organelles to release cytochrome c and other apoptogenic proteins into the cytosol, where cytochrome c binds to the apoptotic peptidase activating factor 1 (Apaf1), then procaspase-9, forming apoptosome to

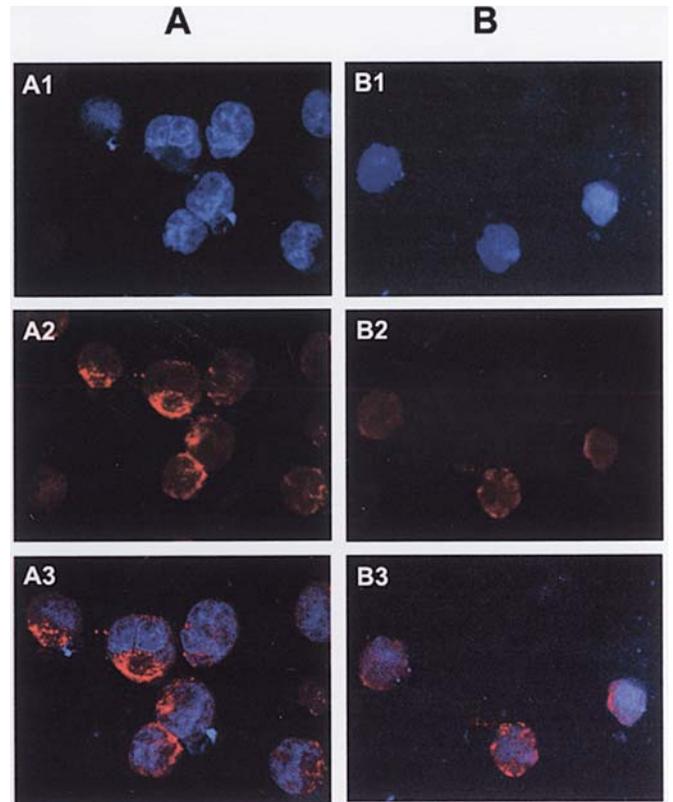


Figure 7. The effects of lycorine on the cytochrome c release from the mitochondria. The KM3 cells were fixed and labeled for cytochrome c (red) and DNA (blue). (A) The untreated cells as the control, (B) The KM3 cells treated with 5  $\mu$ M lycorine for 24 h. The cell nuclei are seen by DNA staining with 4'-6-diamidino-2-phenyl indole (DAPI) (A1 and B1). Cytochrome c became diffusive in most cells (B2), consistent with its translocation into the cytosol and nucleus (B3), whereas it displayed a dotted pattern in the untreated cells, consistent with its location within the mitochondria (A2 and A3). The images were obtained with a confocal laser microscope (x1000).

activate caspase-9, and finally activating downstream effector proteases, such as procaspase-3 (44). In contrast, the extrinsic apoptotic pathway relies on the death receptors of the tumor necrosis factor (TNF) family to bind with death ligands and subsequently activate caspase-8 and -3 for triggering apoptosis (45).

It can be seen that caspases are always at the center of the apoptotic procedure. The Bcl-2 protein family, which consists of anti-apoptotic (e.g. Bcl-2) and pro-apoptotic members (e.g. Bax) (46), is an important regulator of apoptosis. Our results indicate that lycorine can induce the release of cytochrome c from the mitochondria into either the cytoplasm or the cytosol. We found the activation of caspase-8 and -3 to be involved in lycorine-induced apoptosis. Therefore, we concluded that both the intrinsic and extrinsic pathways were involved in the lycorine-induced apoptosis. Furthermore, we have shown that lycorine can increase the Bax protein levels and markedly decrease the Bcl-2 protein levels. To summarize, we have demonstrated that the lycorine-induced apoptosis of the KM3 cells is associated with inducing the release of cytochrome c from the mitochondria, activating caspase-8 and -3, as well as increasing the Bax levels and decreasing the Bcl-2 levels.

The genetic and biochemical investigations of the molecular mechanisms governing the G1 to S phase progression in

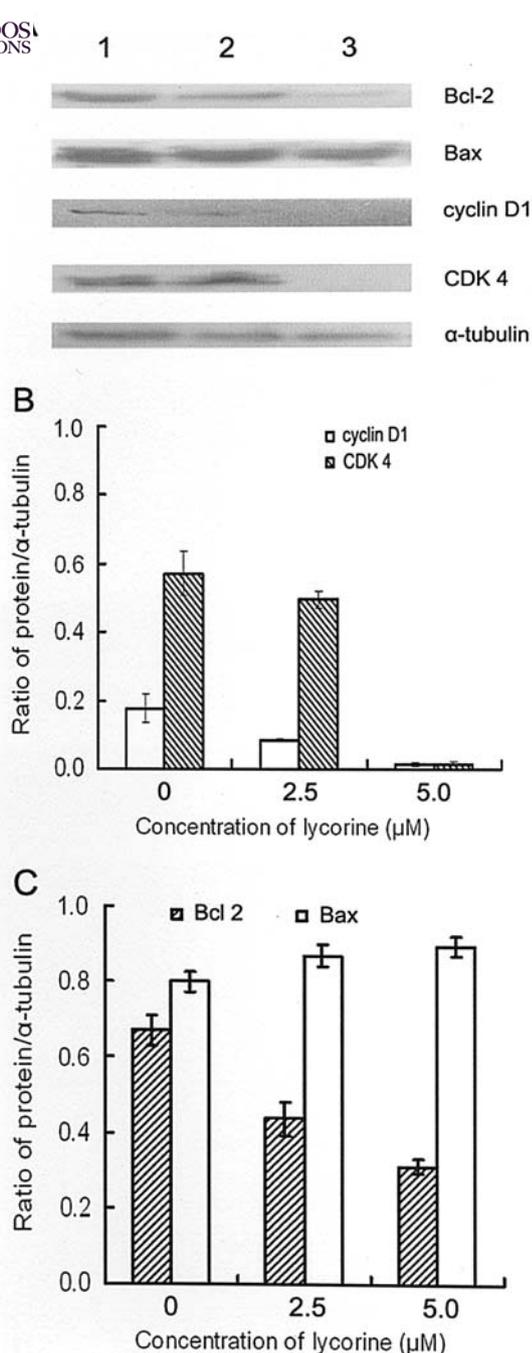


Figure 8. The effects of lycorine on the expression level of Bcl-2, Bax, cyclin D1 and CDK4 in the KM3 cells. The exponentially growing KM3 cells were treated with indicated concentrations of lycorine for 24 h, cell lysates were prepared and the protein level was determined by Western immunoblot analysis. (A) The expression of Bcl-2, Bax, cyclin D1 and CDK4 after lycorine treatment.  $\alpha$ -tubulin was used for the normalization and verification of the protein loading. Lane 1, control; lanes 2 and 3, KM3 cells treated by 2.5, or 5  $\mu$ M lycorine, respectively. (B) The relative level of the cyclin D1 and CDK4 expression after normalization to  $\alpha$ -tubulin. The expression level of both proteins was decreased significantly when the concentrations of lycorine were increased in the culture. (C) The relative level of the Bcl-2 and Bax expression after normalization to  $\alpha$ -tubulin. The expression of Bcl-2 was decreased significantly while the Bax protein level was increased slightly when the concentrations of lycorine were increased in the culture.

mammalian cells have demonstrated a critical role for D-type cyclins and their partners, CDKs 4 and 6 (20,21,47-49). The level of the cyclin D1 protein was found to be a prognostic

marker of disease outcomes for different malignancies. Alterations in different proteins that control the cell cycle progression can be of substantial importance in determining the sensitivities of cells and tumors to some reagents (21). The overexpression of cyclin D1 and CDK4, as well as the dysregulation of growth factors in MM cells, were associated with abnormal proliferation and drug resistance (10,23,49,50). In this study, an increase in the G0/G1 phase cells was observed after the lycorine treatment. Moreover, this study revealed that lycorine suppressed the cyclin D1 and CDK4 gene expression. Therefore, the anti-overexpression of the cyclin D1 and CDK4 gene, could be the mechanism of the lycorine-induced cell cycle arrest of KM3 at the G0/G1 phase.

In the present experiment, we found an increased expression of Bax but a decreased expression of Bcl-2, cyclin D1, and CDK4 proteins in the inhibited cells. Although the order of all these events has not been determined and the relationship of apoptosis and cell arrest is unclear, it can be concluded that a relationship exists between the lycorine-induced cell cycle arrest of KM3 at the G0/G1 phase and both the intrinsic mitochondria and the extrinsic death-receptor pathway of apoptosis through the regulation of the expression of some pro-apoptotic molecules in the KM3 cells. Our results suggest that lycorine could be a potential novel chemotherapeutic compound for the treatment of MM.

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