

Harmine Hydrochloride Induced G2/M Phase Cell Cycle Arrest, Autophagy and Apoptosis in Human Leukemia K562 Cells through Modulation of the MAPK Pathway

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Research

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Abstract

Background

Previous studies have indicated that harmine hydrochloride (HAR-HC) has anti-tumor characteristics. However, its potential impact on human leukemia cells is unknown. In this study, we explored the potential mechanism of HAR-HC effects on human leukemia cells in vitro.

Methods

MTT assay was used to detect cell viability; A flow cytometer was used to analyze the cell cycle; Annexin V-FITC/PI was used to detect cell apoptosis; Western blotting assay was used to analyze the expression of related proteins.

Results

The result of flow cytometry suggested G2/M phase arrest in K562 cells induced by HAR-HC. The expression levels of Cyclin E2, Cyclin D1, Bcl-2, Bcl-xL, Mcl-1, pro-caspase-3, and PARP decreased and the expression levels of Cyclin A2, Cyclin B1, p21, Myt-1, p-cdc2 (Tyr15), cleaved -caspase-3 and cleaved-PARP increased. Moreover, the expression of p-JNK and p-ERK1/2 increased and autophagy was induced in the HAR-HC treatment group. Additionally, HAR-HC facilitated autophagy by activating the ERK1/2 pathway.

Conclusion

HAR-HC induced G2/M phase cell cycle arrest, autophagy and apoptosis by activating the JNK, and ERK1/2 pathways in the human leukemia K562 cells.

Background

Chronic myeloid leukemia (CML) is a malignant tumor that affects blood and bone marrow. The disease is characterized by markedly increased but immature peripheral blood granulocytes. The presence of the Ph chromosome and/or BCR-ABL fusion genes substantially affects the survival and prognosis of patients [1]. Tyrosine kinase inhibitors are the first-line molecular targeted therapy for chronic myeloid leukemia in the clinic [2]. Tyrosine kinase inhibitors can specifically block the binding of ATP to ABL kinase; thus, tyrosine residues cannot be phosphorylated and the proliferation of the BCR-ABL positive cells is inhibited. However, a wide use of tyrosine kinase inhibitors is associated with frequent drug resistance [3] and severe systemic toxicity. Moreover, these drugs cannot completely cure chronic myeloid leukemia. Patients may relapse due to drug resistance or a gene mutation. These factors may limit the use of tyrosine kinase inhibitors. At present, there are no specific targeted therapies for chronic myeloid

leukemia. Allogeneic hematopoietic stem cell transplantation is the only way to cure CML; however, limited availability of suitable donors severely affects the therapeutic options of the patients. Thus, it is necessary to find other therapeutic drugs to improve the cure rate of CML.

Harmine is prevalent in nature. It has various types of pharmacological activators including antimicrobial, antifungal, antitumor, cytotoxic, antispasmodic, antioxidant, antimutagenic, antigenotoxic and hallucinogenic properties [4]. Harmine hydrochloride is a water-soluble nitro-carlin alkaloid with the same properties as harmine. Previous studies have shown that HAR-HC has various antitumor effects. It has been reported that harmine activated intrinsic and extrinsic pathways of apoptosis in B16F-10 melanoma [5]. Additionally, our previous study revealed that harmine induced cell cycle arrest and mitochondrial pathway-mediated cellular apoptosis in SW620 cells by inhibiting the AKT and ERK signaling pathway [6]. Our previous results revealed that harmine hydrochloride triggers the G2 phase arrest and apoptosis in MGC-803 and SMMC-7721 Cells by up-regulating p21, activating caspase-8/Bid, and down-regulating the ERK/Bad pathway [7]. At present, the effect of HAR-HC on chronic myeloid leukemia cells is poorly understood. Therefore, we studied the effect of HAR-HC and its mechanism in K562 cells.

Materials And Methods

Reagents and antibodies.

Harmine hydrochloride (HAR-HC) were purchased from Feida Biochnology (Xi 'an) and dissolved in dimethyl sulfoxide (DMSO). Fetal bovine serum was purchased from Ruite (Guangzhou). Dulbecco's modified Eagle's medium, trypsin and RPI-1640 medium were purchased from GIBCO (USA). 3-(4,5-dimethyl-2- thiazolyl)- 2,5-diphenyl-2-H-tetrazolium bromide (MTT), DMSO, the Annexin V-FITC Kit and propidium iodide (PI) were purchased from Sigma-Aldrich (USA). Antibodies against Bax, ERK, p-ERK, cyclin D₁, cyclinD₃, cyclin E₂, cyclinA₂, cyclin B₁, p21, Myt1, cdc2, p-cdc2, LC3-I/I, PARP, Bcl-2, Bcl-xL, Mcl-1, pro-caspase-3, cleaved-caspase-3 and GAPDH were purchased from Cell Signaling Technology (CST, USA). z-DEVD-fmk, U0126 and chloroquine (CQ) were purchased from Selck (USA).

Cell lines and cell culture.

The human cell lines human leukemia K562 and normal liver epithelial LO2 cells were obtained from Department of Biochemistry, Basic Medical College, Jinan University (Guangdong, China). LO2 cells were cultured in high glucose DMEM containing 10% FBS. Chronic leukemia K562 and lymphoma Raji cells were cultured in RPMI-1640 medium with 7% FBS. The cells were maintained at 37°C in a humidified incubator with 95% air and 5% CO₂.

Cell viability assay.

Cells (6×10^3 /ml) were seeded in 96-well plates overnight and then treated with various concentrations of HAR-HC (0, 5, 10, 20, 40 and 80μM). After incubation of 24,48 or 72 h, 90 μL fresh medium without FBS and with 10 μL of MTT was added to each well for 4 h at 37°C. Then, discarded the supernatant by centri

and 150 µL DMSO was added to each well. Finally, a microplate reader was used to measure the absorbance at 570 nm in each well.

Cell cycle analysis.

Cells were seeded at a density of 5.0×10^5 per well in six-well plates. The cells were exposed to various concentrations of HAR-HC (0, 10, 20 and 40 µM) for 24 h. The cells were harvested, washed twice with cold PBS and fixed with 70% cold ethanol overnight at 4°C. Cells were subjected to the cell cycle distribution analysis after 30 min of incubation with RNase and PI in the dark. A flow cytometer was used to analyze the samples.

Cell apoptosis analysis.

Cells were seeded at a density of 5.0×10^5 per well in six-well plates. After 24 h, the cells were exposed to various concentrations of HAR-HC (0, 10, 20 and 40 µM) for 24 h. The cells were harvested, washed twice with cold PBS. Then, 5µL of Anexinn V-FITC/PI and 5µL PI were added to the cells and incubated for 15 min in the dark at room temperature following the manufacturer's protocol. A flow cytometer was used to analyze the samples.

Western blotting assay.

Cellular proteins were extracted after treatment with a cell-lysis buffer which containing proteinase inhibitors on ice for 30 min and the samples were centrifuged at 12,000 rpm at 4°C for 15 min. Then, the total protein was quantified using a BCA protein assay kit according to the manufacturer' instructions. The samples were subjected to 10% or 15% SDS-PAGE, and the protein bands were transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% dried nonfat milk, the membranes were incubated overnight at 4°C with an appropriate primary antibody. Then, the membranes were washed three times in Tris-buffered saline with tween 20 (TBST) and incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. After three washes in TBST, the bands on the membranes were visualized with an ECL chemiluminescence reagent and X-ray film (Kodak, Rochester, NY, USA) as described previously.

Statistical analysis.

Statistical analysis was done by GraphPad software, version 5. Data are presented as the means \pm standard error of the means (SEM) or standard Deviation (SD). Dunnett-t test was applied to assess the statistical significance. p value < 0.05 were considered significant.

Results

Harmine hydrochloride inhibited the proliferation and induced the G2/M cell cycle arrest in K562 cells.

HAR-HC is a derivative of the natural beta-carboline alkaloid with a better water solubility than harmine (Fig. 1A). We examined the effects of HAR-HC on cell viability in K562, Raji and LO2 cells. After cells were exposed to indicated concentrations (0, 5, 10, 20, 40 and 80 μ M) of HAR-HC for various times (24, 48 and 72 h), it demonstrated a remarkable inhibition effect on the proliferation of K562 cells with the IC₅₀ values ranged from 30 μ M to 70 μ M, and the IC 50 values of Raji cells ranged from 30 μ M to 35 μ M of (Fig. 1B, 1C, Table 1), while the IC 50 values of HAR-HC on LO2 cells at 24 h and 48 h were > 80 μ M (Table.1). These results showed that HAR-HC inhibited K562 and Raji cells' proliferation.

Table1. The IC50 values of LO2 cells and cancer cells after treated with Harmine-hydrochloride ($\bar{x} \pm S$)

Cells	IC50 values (μ M)		
	24h	48h	72h
LO2	>80	>80	63.09±11.35
K562	74.12± 4.00	16.48± 1.84	17.87± 2.19
Raji	33.99± 7.99	30.38± 1.65	35.80± 4.83

K562, Raji and LO2 cells were treated with HAR-HC for various times(24, 48 and 72 h),IC50 was detected using the MTT assay.

In addition, we analyzed the cell cycle distribution by flow cytometry. Compared with the control, HAR-HC treatment resulted in a decrease in the G1 phase and an accumulated population in the G2 phase at 24 h, while the fraction of the S phase cells was approximately the same in the untreated and treated groups (Fig. 2A and 2B). We also found that HAR-HC treatment decreased the expression levels of cyclin D₁, cyclin D₃ and cyclin E₂, while the levels of cyclin A₂ and cyclin B₁ increased (Fig. 2C). And the expression levels of the p21, Myt1, p-cdc2 (Tyr¹⁵) proteins were increased (Fig. 2D). Thus, we concluded that HAR-HC increased the protein levels of p21 and Myt1 leading to the G2/M phase arrest in K562 cells.

Harmine hydrochloride induced apoptosis in K562 cells.

To further investigate the potential mechanism of HAR-HC regulation of K562 cells, we detected apoptotic cells by the Annexin V-FITC/PI double staining assay. The results showed that the apoptosis in K562 cells increased with increasing concentrations of HAR-HC (Fig. 3A). Hence, we determined whether mitochondria-associated apoptosis pathway was involved in the HAR-HC group. The expression levels of the anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1 were significantly reduced. However, the expression of Bax was not significantly influenced (Fig. 3B). After treatment with HAR-HC for 24 h, the expression levels of cleaved caspase-3 and cleaved PARP were increased in K562 cells (Fig. 3C). To further investigate whether HAR-HC-induced cell apoptosis was caspase dependent, z-DEVD-fmk, a caspase 3 inhibitor was used. The results showed that z-DEVD-fmk reversed the changes in the expression levels of cleaved-

caspase 3 and cleaved-PARP. These results indicated that HAR-HC induced apoptosis in K562 cells through the mitochondria-associated apoptosis pathway (Fig. 3D).

HAR-HC induced autophagy in K562 cells.

Autophagy is the cellular pathway the clearance of ubiquitinated proteins in the lysosomes during nutrient deprivation or cellular stress [8]. As shown in Fig. 4A, the downregulation of p62 and the increase of LC3-II conversion indicated that HAR-HC upregulated the formation of autophagosomes in a dose-dependent manner in K562 cells. To confirm that autophagy is induced by HAR-HC, CQ, an inhibitor of autophagy was added to K562 cells for 1 h prior to HAR-HC exposure. Western blot showed that the expression levels of p62 and LC3-II were reversed by CQ (Fig. 4B), suggesting that HAR-HC induced autophagy in K562 cells.

Autophagy played a protective effect in K562 cells.

We found that the expression levels of cleaved -caspase-3 and cleaved -PARP were increased in the HAR-HC group and the expression levels of cleaved-caspase-3 and cleaved-PARP were even further increased in HAR-HC and CQ combination group (Fig. 4C). These results showed that HAR-HC-elicited autophagic response played a protective effect, and inhibition of autophagy could sensitize K562 cells to HAR-HC.

HAR-HC-treated K562 cells activated the MAPK signaling pathway.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases mainly involved in embryonic development, cell differentiation, cell proliferation and cell death[9–11]. ERK was considered to be the classical MAPK pathway participating in cell growth, proliferation and differentiation. However, the role of ERK1/2 in inducing cell apoptosis remains controversial. The results showed that the expression levels of p-JNK and p-ERK were increased in K562 cells after HAR-HC treatment (Fig. 5A).

Activating of ERK1/2 pathway induced autophagy, inhibit apoptosis in K562 cells. To verify between activation of ERK pathway and autophagy in HAR-HC treatment cells, a MEK/ERK- specific inhibitor U0126 was used. The results showed that the expression levels of p-ERK1/2 were increased in the HAR-HC group and were reversed in HAR-HC and U0126 combination group (Fig. 5B). We also found that the expression of p62 was downregulated and the expression of LC3-II was upregulated in the HAR-HC group; the downregulation of p62 and the upregulation of LC3-II were reversed in the HAR-HC and U0126 combination group (Fig. 5B). That meant activation of ERK pathway induced autophagy, and inhibition of ERK pathway blocked autophagy in K562 cells.

We also investigated the relationship between the activation of ERK pathway and apoptosis in K562 cells treated with HAR-HC. As shown in Fig. 5C, western blotting assay revealed that the expression levels of cleaved-caspase-3 and cleaved-PARP were increased in the HAR-HC group and the expression of cleaved-caspase-3 and cleaved-PARP were even further increased in the HAR-HC and U0126 combination group. These results demonstrated that apoptosis was enhanced by inhibition of ERK1/2 pathway, suggesting

that activating of the ERK1/2 pathway reduced apoptosis and promoted cell survival in HAR-HC-treated K562 cells.

Discussion

At present, conventional chemotherapy is one of the main methods of cancer treatment. Most chemotherapeutic drugs have a low response rate and poor efficacy in the clinic. More importantly, severe toxicity and resistance of chemotherapy reduce the life quality of patients and the effectiveness of chemotherapeutic drugs. In recent years, numerous natural products and their derivatives have received considerable attention from pharmacologists and chemists studying the use in the treatment of tumors [12–14]. In this study, the anticancer activity of HAR-HC was detected. The results showed that HAR-HC induced the G2/M phase arrest and caspase-dependent apoptosis and autophagy in K562 cells through modulation of the MAPK signaling pathway.

In this study, a G2/M phase arrest treated with HAR-HC was detected in K562 cells by flow cytometry. We demonstrated that HAR-HC decreased the expression levels of Cyclin D1, D3 and Cyclin E2 and increased the expression levels of Cyclin B₁, Cyclin A₂, Myt-1, p21 and p-cdc2^{Tyr¹⁵} in K562 cells. Up-regulation of p21 inhibited the activation of the cdc2 and Cyclin B₁ complexes, while Myt-1 reduced activity of cdc2 by phosphorylating Tyr¹⁵ leading to G2/M phase in K562 cells.

ERK1/2 signal pathways regulate cell growth, proliferation and differentiation and the JNK, p38 signal pathways play important roles in stress responses including inflammation and apoptosis[15–18]. We detected that the expression levels of anti-apoptosis proteins (Bcl-2, Bcl-xL and Mcl-1) were down-regulated and enhanced the ratio of Bax/Bcl-2, Bax/Bcl-xL. HAR-HC promoted activation of caspase-3 and PARP in K562 cells. While we added z-DEVD-fmk, a caspase 3 inhibitor, before the treatment of HAR-HC, and the expression levels of cleaved-caspase 3 and cleaved-PARP were reversed. The results showed that HAR-HC induced apoptosis via caspase-dependent manner. Studies have shown that JNK activation can induce apoptosis[7, 9]; moreover, SP600125, a JNK-specific inhibitor, partially but significantly rescued the cells from death and significantly repressed caspase-3 activity [19, 20]. Some studies demonstrated that ERK1/2 is also important for induction of apoptosis [21–23]. However, recent studies demonstrated that ERK1/2 can suppress apoptosis via phosphorylation of the pro-apoptotic Bcl-2 family proteins [17, 18, 23]. To determine the detailed mechanism of HAR-HC induction of apoptosis, we investigated the role of HAR-HC in MAPK signaling, and found that HAR-HC treatment activated JNK and ERK in K562 cells.

Autophagy is an intracellular metabolic process. In the progress of autophagy, cytoplasmic LC3- κ binds to phosphatidyl ethanolamine and is transformed into LC3- π . In the present study, we demonstrated that HAR-HC treatment increased the expression of LC3- π /LC3- κ ratio and decreased the express levels of p62. These results suggested that HAR-HC could induce autophagy in K562 cells. Autophagy plays a dual role in the survival and death of cancer cells [24, 25]. To further confirm the relationship between pro-survival and pro-apoptotic effect of HAR-HC inducing autophagy in K562 cells, an autophagy inhibitor (CQ) was

used. The results showed that CQ treatment increased apoptosis in the CQ and HAR-HC combination group. This result revealed that autophagy may play a pro-survival role in HAR-HC-induced apoptosis. ERK signaling pathway is involved in regulation of autophagy and apoptosis [12, 16]. In the experiment, we found that autophagy was inhibited and apoptosis was increased in U0126 and HAR-HC combination group. ERK1/2 activation and autophagy played a pro-survival role in the HAR-HC-induced K562 cells.

Conclusion

In conclusion, our research demonstrated that HAR-HC induced G2/M phase arrest in K562 cells via the up-regulation of p21, activation of Myt1 and phosphorylation of cdc2. Additionally, HAR-HC induced mitochondria-related apoptosis and autophagy by modulating the expression of Bcl-2 protein family, activating the JNK and ERK1/2 signaling pathways. ERK1/2 activation reduced apoptosis and increased autophagy in HAR-HC treated K562 cells.

Abbreviations

HAR-HC: Harmine hydrochloride; CML: Chronic myeloid leukemia; DMSO: Dimethyl sulfoxide; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; PI: Propidium iodide; CQ: Chloroquine; PVDF: Polyvinylidene difluoride; TBST: Tris-buffered saline with tween 20; BCA: Bicinchoninic acid; IC50: Fifty percent inhibitory concentration; PARP: Poly ADP-ribose polymerase; MAPK: Mitogen-activated protein kinase pathway; ERK: Extracellular regulated protein kinases; JNK: C-Jun N-terminal kinase

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Zhilong Liu and Peng ZhangConceptualization, Methodology, Software. Zhilong Liu, Peng Zhang and Lin-lin LvData curation, Writing- Original draft preparation. And Na Zhao and Ziyu Li: Visualization, Investigation. Qiang Li and Yuxia YanSupervision.

Na Zhao and Zhongnan Wu: Software, Validation. Wen-dan Chen and Jian-wei Jiang: Writing- Reviewing and Editing.

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Figures

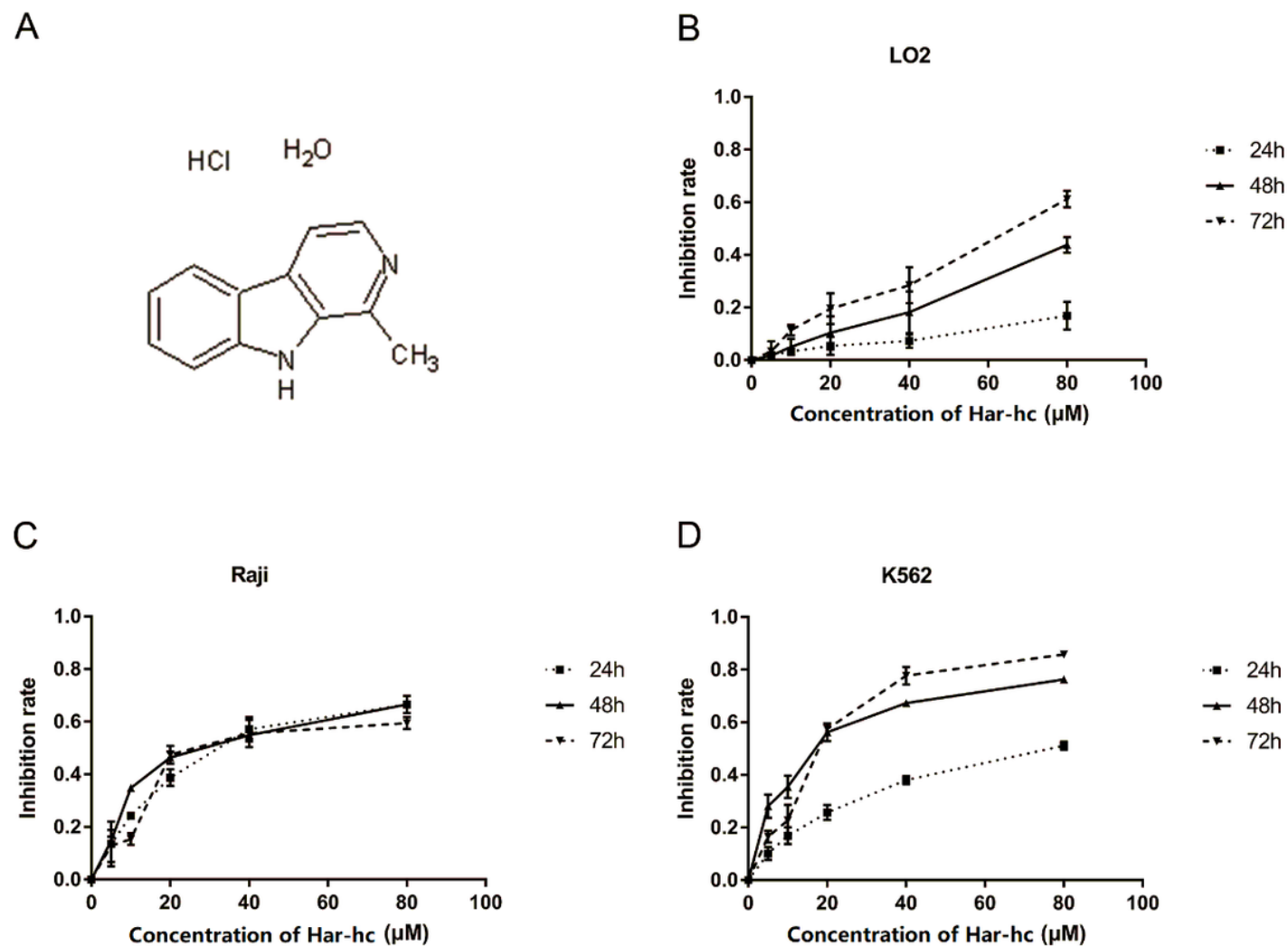


Figure 1

Inhibition of proliferation K562 cells by harmine hydrochloride. (A) Chemical structure of harmine-hydrochloride. (B) Cytotoxic effects of HAR-HC on LO2 cells. Cells were cultured at 6,000 cells per well in a 96-well plate, exposed to different concentrations of HAR-HC and incubated for 24 h, 48 h and 72 h. Growth inhibition was detected using the MTT assay. The results are representative of three independent experiments. (C) Cytotoxic effects of HAR-HC on Raji cells. Cells were cultured at 6,000 cells per well in a 96-well plate, exposed to different concentrations of HAR-HC and incubated for 24 h, 48 h and 72 h. Growth inhibition was detected using the MTT assay. The results are representative of three independent

experiments. (D) Cytotoxic effects of HAR-HC on K562 cells. Cells were cultured at 6,000 cells per well in a 96-well plate, exposed to different concentrations of HAR-HC and incubated for 24 h, 48 h and 72 h. Growth inhibition was detected using the MTT assay. The results are representative of three independent experiments.

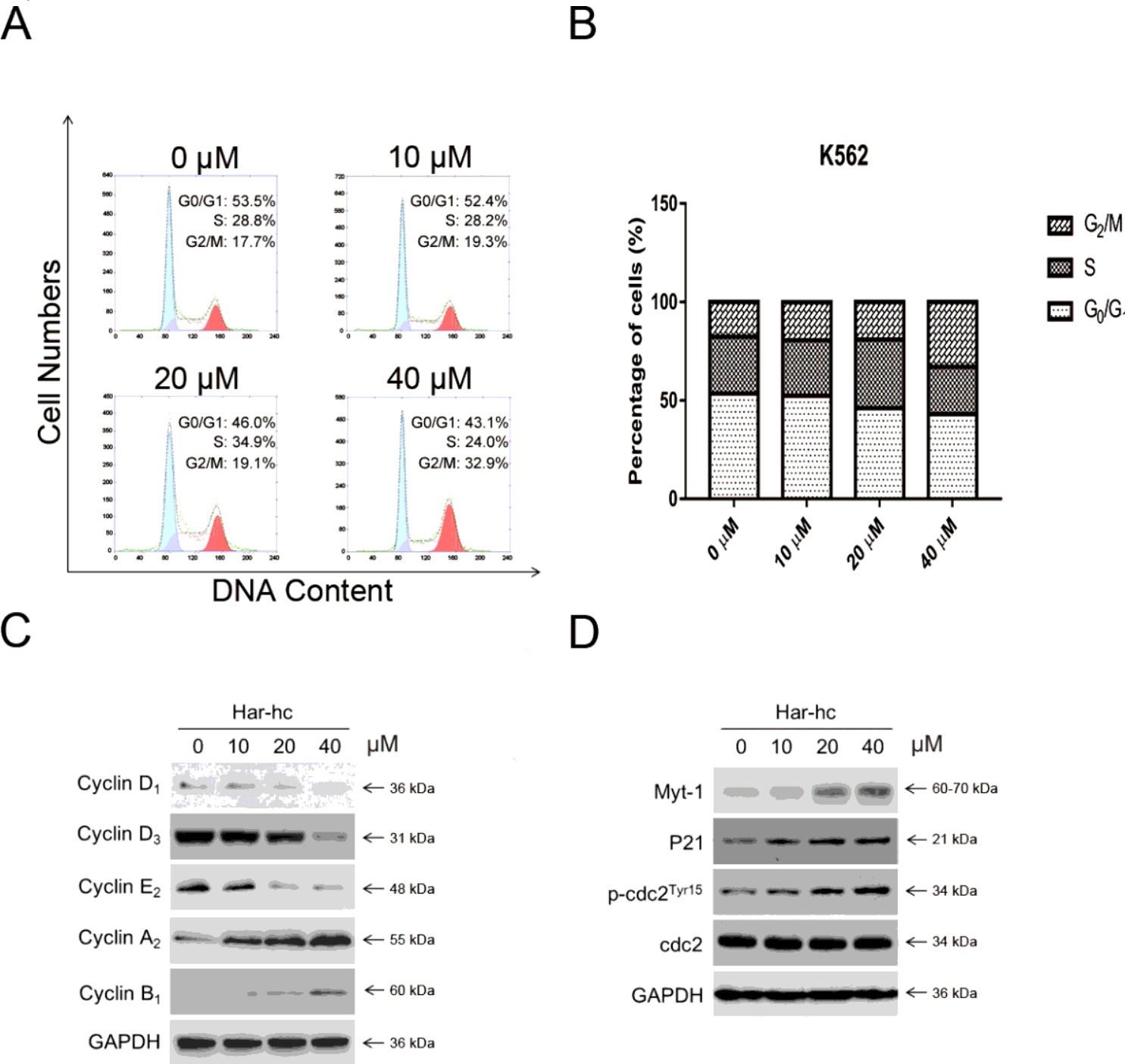
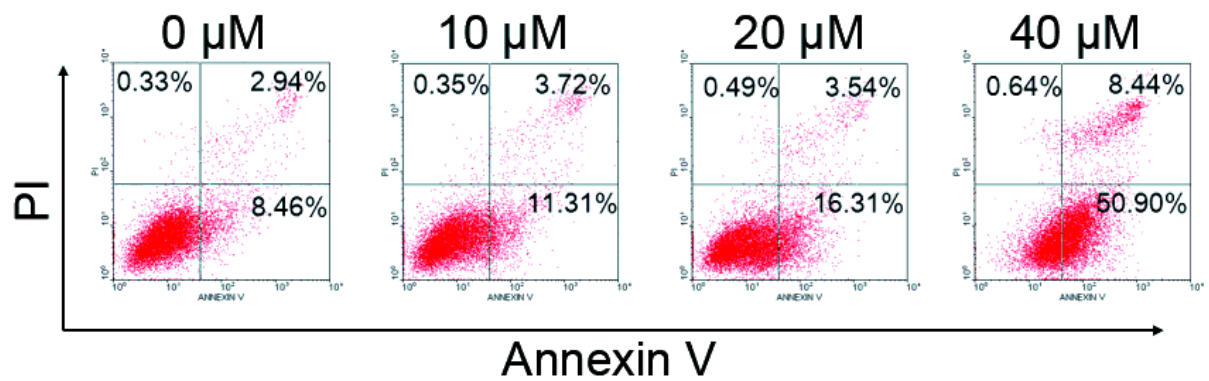


Figure 2

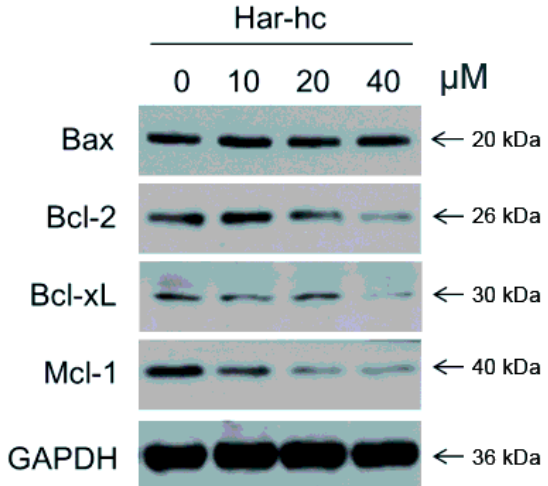
HAR-HC treatment in K562 cells resulted in and G2/M arrest. (A) cells were treated with different concentrations of HAR-HC for 48 h, and DNA content was detected by flow cytometry. (B) a stacked bar chart was used to indicate the percentage of cells in G0/G1, S, G2/M phases. (C and D) After 24 h incubation with indicated concentrations of HAR-HC, western blot analysis was conducted with

antibodies against cell cycle-associated proteins. Data represent three independent experiments. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is shown as a loading control.

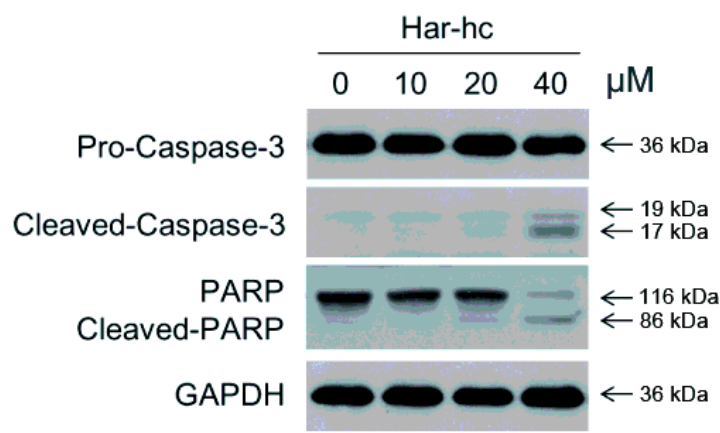
A



B



C



D

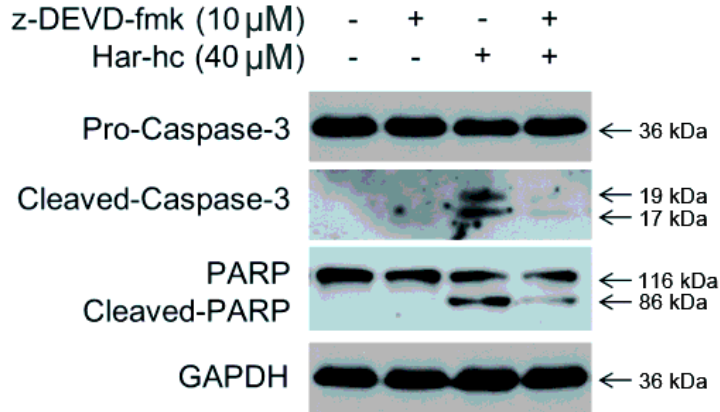


Figure 3

HAR-HC induced apoptosis in K562 cells. (A) The apoptosis rates at various concentrations (0,10,20 and 40 μM) of HAR-HC were assessed by flow cytometry after AnnexinV-FITC/PI staining. (B and C) The expression levels of apoptosis-related proteins were detected by western blot after 24 h incubation with

HAR-HC without of a caspase inhibitor (z-DEVD-fmk). (D) The expression levels of apoptosis-related proteins were detected by western blot after 24 h incubation with HAR-HC with a caspase inhibitor (z-DEVD-fmk).

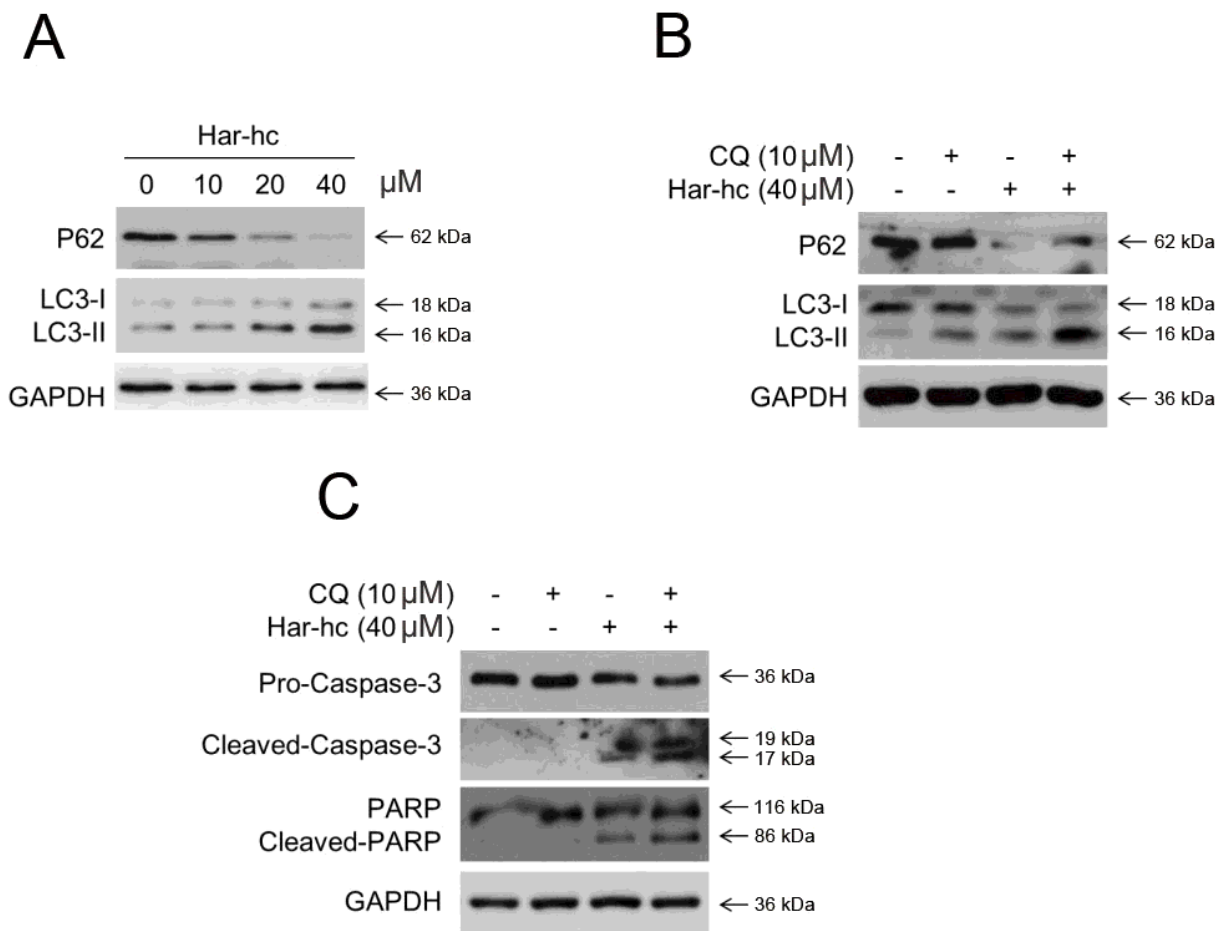


Figure 4

HAR-HC induced autophagy in K562 cells . (A) K562 cells were incubated with various concentrations of harmine-hydrochloride for 24 h. The expression levels of autophagy-related proteins were measured by western blot. (E) The expression levels of the autophagy-related proteins were detected by western blot in cells treated with CQ (10 μM). (C) The expression levels of the autophagy-and apoptosis-related proteins were detected by western blot in cells treated with CQ (10 μM).

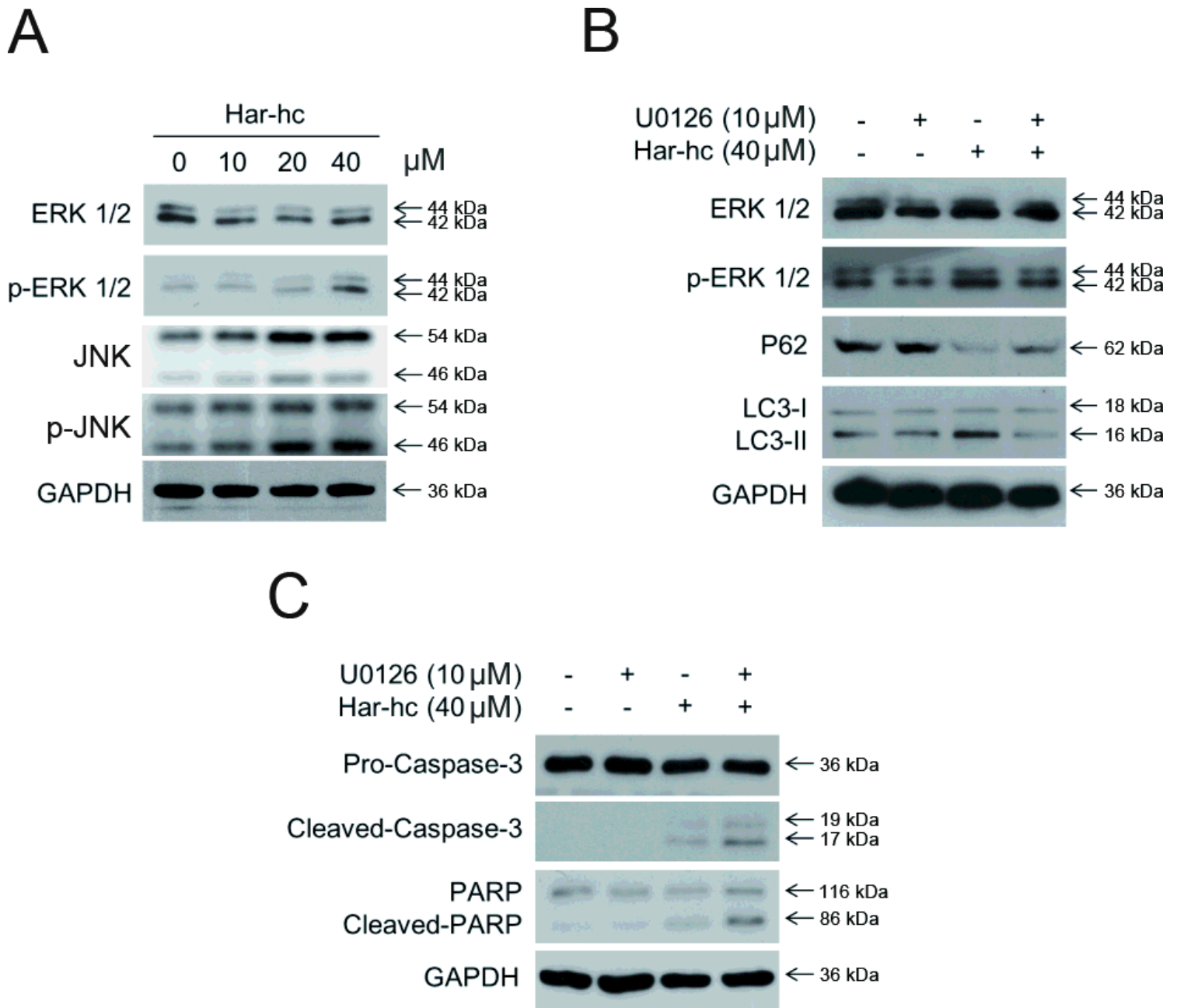


Figure 5

MAPK expression in K562 cells treated with HAR-HC. (A) K562 cells were incubated with various concentrations of harmine-hydrochloride for 24 h. The expression levels of JNK, p-JNK, ERK1/2 and p-ERK1/2 proteins were measured by western blot. (B) The expression levels of ERK1/2 signaling-related proteins and autophagy-related proteins were detected by western blot in cells treated with a highly selective MEK/ERK inhibitor U0126. (C) The expression levels of apoptosis-related proteins were detected by western blot in cells treated with a highly selective MEK/ERK inhibitor U0126.