# Lycorine Hydrochloride Inhibits Cell Proliferation and Induces Apoptosis Through Promoting FBXW7-MCL1 Axis in Gastric Cancer

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**Research**

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# Abstract

**Background:** Lycorine hydrochloride (LH), an alkaloid extracted from the bulb of the lycorisaceae plant, is considered to have anti-viral, anti-malarial and anti-tumorous effects. At present, the underlying mechanisms of LH in gastric cancer remain unclear. MCL1, an anti-apoptotic protein of BCL2 family, is closely related to drug resistance of tumor. Therefore, MCL1 is considered as a potential target for cancer treatment.

**Methods:** The effect of LH on gastric cancer was assessed in vitro (by MTT, BrdU, western blotting…) and in vivo (by immunohistochemistry H&E).

**Results:** In this study, we showed that LH has an anti-tumorous property by down-regulating MCL1 in gastric cancer. Besides, we unveiled that LH reduced the protein stability of MCL1 by up-regulating ubiquitin E3 ligase FBXW7, which induced S-phase cell cycle arrest and triggered apoptosis of gastric cancer cells. Meanwhile, the results also showed that LH could induce apoptosis of the BCL2-drug-resistant-cell-lines. Moreover, PDX (Patient-Derived tumor xenograft) model experiment showed that LH combined with HA14-1 (inhibitor of BCL2), had a more significant therapeutic effect for gastric cancer.

**Conclusions:** Together, the efficacy shown in our data suggests that lycorine hydrochloride is a promising anti-tumorous compound for gastric cancer.


# Background

As a malignant tumor originating from the epithelium of gastric mucosa, gastric cancer affects the health of nearly 1 million individuals every year. The high mortality rate associated with gastric cancer (nearly 800,000 deaths per year) is mainly due to late diagnosis and limited treatment options [1, 2]. Although some progress has been made in the prevention, early diagnosis and effective treatment of gastric cancer, the prognosis of gastric cancer remains to be improved [3–5]. For approximately 80% of patients, the diagnosis is lagged and frequently relapses after surgery. Standard surgical resection is not ideal for advanced gastric cancer treatment. Therefore, the screening of new drugs is particularly urgent [6]. Besides, another real problem we have to face is that multi-drug-resistance has become one of the biggest obstacles to the success of cancer chemotherapy [7, 8]. Accordingly, the development of effective inhibitors of drug resistance targets is also insistent.

Multiple shreds of evidence have shown that chemo-resistance is closely related to the intrinsic apoptosis regulators [9–12]. The BCL2 gene family proteins are essential regulators of apoptosis and play vital roles in maintaining the physiological differentiation of cells and the dynamic balance of cell numbers. The members of the BCL2 family have conservative BCL2 homology (BH) domain sequences (BH1-BH4). The whole family is divided into two groups, with the entirely opposite functions. The pro-survival proteins, including BCL2, BCL-XL, BCL-W, BFL-1/A1 and MCL1, promote cell survival. Whereas, the pro-apoptotic proteins,including BIM, BID, PUMA, NOXA, BAD, BMF, HRK and BIK, promote cell death [13, 14]. When the apoptosis signal is triggered, a subset of pro-apoptotic proteins (like BIM, NOXA and PUMA)

which have the BH3-only region, cause BAK and BAX homologous oligomerization and form pores in the mitochondrial membrane, leading to the release of Cytochrome C into the cytosol and further triggering apoptosis [15]. In this process, anti-apoptotic proteins (like BCL2, BCL-XL and MCL1) dynamically regulate apoptosis by binding or sequestering with the BH3-only domain proteins [16].

Considering the critical functions of the BCL2 family in cancer therapy, researchers have developed a large number of small molecule inhibitors over the past 10 years. ABT-737, the first BH3 mimetic inhibitor of BCL2, BCL-XL and BCL-W, exhibits favorable single-agent anti-tumorous activity in various tumor models [17]. Subsequently, ABT-263 (the upgraded products of ABT-737), as well as BM 1197, S44563, BCL2 32, and AZD4320, several inhibitors of BCL2 and BCL-XL, were also reported to inhibit cancer progression successively [17, 18]. Further, the effects of mono-selective BCL2 inhibitors such as ABT-199 (also known as Venetoclax) and S55746 (also called BCL201 or Servier-1) were also reported in cancer research [19]. In previous clinical studies, ABT-263 showed single-drug efficacy in a variety of tumor types [20, 21]. However, amplification of MCL1 is a potent factor of resistance to ABT-737 and ABT-263. Besides, agents that promote the degradation of MCL1 synergistically could induce apoptosis [22]. Many BCL2 inhibitors have been developed and clinically tested, but MCL1 inhibitors are not available in clinical trials [23]. In addition, MCL1 is an essential cause of resistance to radiation and chemotherapy, including inhibitors targeting the other BCL2 family members [24]. For example, BCL2 selective inhibitor ABT-199 has shown high efficacy in the treatment of chronic lymphocytic leukemia (CLL), but it cannot induce apoptosis in certain tumor cell lines with MCL1 amplification, in the recently report [25]. Therefore, the development of effective MCL1 inhibitors has become an urgent need for clinical treatment.

Lycorine hydrochloride (LH), a derivative of lycorine, is an isoquinoline alkaloid extracted from *Lycoris*. According to previous reports, lycorine has a variety of pharmacological activities including anti-tumor, anti-virus, anti-inflammatory, anti-malaria, inhibition of acetylcholinesterase activity, etc. [26–28]. It has been reported that lycorine and its derivatives have significant inhibitory effects on leukemia, lymphoma, melanoma, esophageal cancer, breast cancer, ovarian cancer, prostate cancer, etc. [25]. So far, the existing evidence showed that LH has stronger therapeutic effect on tumor cells than normal cells [29]. However, LH has rarely been reported in gastric cancer. Therefore, it is necessary to study the impact of LH on gastric cancer and explore the underlying mechanisms.

# Materials And Methods

### Reagents and antibodies

The MCL1 (16225-1-AP), HA(51064-2-AP), Alpha Tubulin (11224-1-AP) and FBXW7 (28424-1-AP) antibodies were purchased from Proteintech Group (USA), and antibodies CDK1( #77055), CDK2 (#78B2), Cleaved Caspase 3 (#14220), Cleaved Caspase 9 (#52873), Cleaved PARP (#5625), PARP (#9542), Bim (#2933), BAX (#5023) BCL2 (#15071) were purchased from Cell Signaling Technology (USA). 5-Bromo-2-deoxyUridine (BrdU) (ab8152) and Ki67 (ab15580), β-TrCP (ab71753), HUWEI/mule (ab70161) antibodies were purchased from Abcam (USA). The One Step TUNEL Apoptosis Assay Kit (C1089) was purchased

from Beyotime (China). Cycloheximide (C7698), 3-(4, 5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide (MTT; M5655), DMSO (D5879), and MG132 (M7449) were purchased from Sigma-Aldrich (USA). Lycorine hydrochloride was purchased from MUST BIO-TECHNOLOGY (Cheng Du, China). Alexa Fluor 488 goat anti-rabbit IgG (H+L) (35552) was purchased from Invritrogen (USA). The puromycin (A1113803) was purchased from Life Technologies (New York, USA). The transfection reagent Lipofectamine™ 2000 was obtained from Thermo Fisher Scientific (New York, USA). HRP goat anti-mouse (Cat. No: 5220-0341) and goat anti-rabbit antibodies (Cat. No: 5220-0336) were purchased from Seracare company (USA). Annexin V-APC (2005128) and propidium iodide (2048964) were obtained from Invitrogen (USA). The propidium iodide (2031727) for cell cycle was purchased from Invitrogen (USA).

### Cell Culture

MKN-45, SGC-7901 and 293-FT cell lines were purchased from American Type Culture Collection (ATCC, USA). MKN-45-R and SGC-7901-R cell lines were obtained from our laboratory. All cells were cultured in 1640 medium (with 10% fetal bovine serum, 1% Penicillin-Streptomycin Solution) at 37 °C in a humidified atmosphere containing 5% $CO_2$.

### Cell viability (MTT), BrdU staining assays, western blotting analysis and qRT-PCR

Cell viability (MTT), BrdU staining assays, western blotting assays and qRT-PCR assays were analyzed as previously described [30].

### Flow cytometry

(a) Cell cycle analysis: MKN-45 and SGC-7901 cells were harvested after added with LH for 48h, and fixed in 70% ethanol at 4°C for 24h. Washed with PBS, the cells were added into 50 μg/mL RNase A and 1% propidium iodide for 35 minutes at 37 ℃. Subsequently, the BD Accuri C6 flow cytometer and FlowJo software were used to analyze the change of DNA.

(b) Cell apoptosis analysis: MKN-45 and SGC-7901 cells were harvested after being treated with LH for 48h. After being washed with 1x Bingding buffer, the cells were incubated with Annexin V-APC (0.5%) and propidium iodide (1%) at room temperature for 20 min. Subsequently, the BD Accuri C6 flow cytometer and FlowJo software were used to analyze the change of apoptosis following the manufacturer's instructions.

### TUNEL experimental analysis

Twenty thousand cells were evenly spread on the 24-well cell culture plate. After 24 hours, LH (20 μm) and DMSO were added respectively, incubated for 48 hours. Washed it with PBS once. Then 4% PFA fixed the cells for 30 minutes. Washed again with PBS. Added PBS containing 0.5 % Triton X-100 and incubated at room temperature for 5 minutes. Cleaned it with PBS twice. Added the prepared TUNEL test solution (50 μL) every well, incubated at 37 ℃ in the dark for 60 minutes. Then washed three times with

PBS. It was observed by fluorescence microscope after sealing with anti-fluorescence quenching solution. The excitation wavelength of Cy3 is 550 nm, and the emission wavelength is 570 nm (red fluorescence).

## Transfection and infection

The interference plasmid shMCL1 (#1; #2) and the negative control (SHC002) were purchased from Sigma-Aldrich (USA). The MCL1-overexpression plasmid (pCDH-CMV-MCS-EF1-copGFP-MCL1) was purchased from YouBio (Changsha, China) [19]. Transfection and infection were carried out as manufacturer's instructions. Firstly, the liposomes and the packaging plasmids (PLP1, PLP2, VSVG, target plasmids: shMCL1 or OE-MCL1) were transferred into 293-FT cells. The virus was harvested 48 hours later. Gastric cancer cell lines MKN-45, SGC-7901 were infected with the harvested virus.

## Subcutaneous tumour xenografts (CDX model)

Experiments in vivo were carried out with the approval of the Committee for Animal Protection and Utilization of Southwest University. According to the Guidelines for Animal Health and Use (Ministry of Science and Technology, China, 2006), all experiments were conducted orderly. Purchased from Huafukang Biotechnology Co., Ltd. (Beijing, China), six five-week-old female nude mice were raised and observed in SPF room for a week to adapt to the new environment. On June 20, 2019, SGC-7901 cells ($1 \times 10^6$ cells per mouse) suspended in 0.1 ml serum-free RPMI-1640 were subcutaneously inoculated on the left and right upper back of mice. In order to alleviate the pain of mice, we used the isoflurane for nasal anaesthesia before subcutaneous injections. Isoflurane can make the mice enter anesthesia state faster and recover quickly. After the anesthesia stops, the mice commonly wake up within 2 minutes, and the control of anesthesia depth was very easy. If the mice were found to be out of condition during the operation, the anaesthesia machine would be shut off immediately, and the mice would be rescued quickly. Hence, the safety of mice was guaranteed. Isoflurane can be completely discharged from the alveoli through respiration without affecting metabolism in vivo, and has no effect on the experimental results. In addition, isoflurane is widely used in animal experiments in the world. The mouse anaesthesia system was purchased from Reyward Life Technology Co., Ltd. (Shenzhen, China). All experiments were performed on a sterile workbench of an SPF room [31]. Before and after subcutaneous injection, 75% medical alcohol was used to disinfect the epidermis of mice. After one week injection, they were randomly divided into two groups, which were respectively treated with DMSO and LH (30mg / kg) once a day for 16 days. During this period, tumor volume [tumor volume= (length × width $^2$)/2] was measured every two days under strict and standardized feeding conditions. Before tumor collection, nasal anesthesia (isoflurane) was used in mice to relieve pain. Then, the mice were killed by cervical dislocation. The tumor was removed, and the weight was recorded. The bodies were frozen at -20°C before transferring to Laibite Biotech Inc. (Chongqing, China) for incineration. Finally, the tumor was photographed and recorded, which will be used for subsequent immunohistochemistry experiments.

## PDX experiment

Purchased from Huafukang Biotechnology Co., Ltd. (Beijing, China), nine five-week-old female nude mice were raised and observed in SPF room for a week to adapt to the new environment. On November 10, 2019, with the consent of the patient's family, the GAM-AD tumor mass from The Ninth People's Hospital of Chongqing was cut into even 2x2x2mm pieces and suspended in 0.1ml serum-free RPMI-1640, and planted into subcutaneous tissue of mice in equal volume. The whole process is consistent with the previous subcutaneous tumour xenografts experiment. Two weeks after operation, they were randomly divided into three groups, respectively treated with DMSO, LH (30mg/kg) and LH (30mg/kg)+HA14-1 (2.5mg/kg) once a day for 16 days. In addition, after one week operation, the weight of the mice was recorded every four days. The process of tumor collection was also consistent with the aforementioned subcutaneous tumour xenografts experiment. The tumor weight was recorded, and the tumor was photographed and recorded, which will be used for subsequent immunohistochemistry experiments.

### Immunoprecipitation (Co-IP)

Protein A/G Magnetic Beads (HY-K0202) were purchased from MCE (Monmouth Junction, NJ, USA). Cells treated with LH or DMSO were lysed and collected. Then, operate according to the instructions.

### Ubiquitination assay

Firstly, the HA-Ub plasmid was transiently transferred into 293-FT. Then 293-FT cells were harvested before incubating with proteasome inhibitor MG132 (50 μg/ml) from Selleck (Houston, USA) for 6 h. The harvested cells were lysed by the IP lysis solution. Subsequently, incubated with anti-MCL1 (1%) or IgG at 4 ℃ for overnight. The second day, the Protein A/G Magnetic Beads were added following with the instruction. Then, the proteins adsorbed from magnetic beads were analyzed by western blotting. HA tag antibody (51064-2-AP) from Proteintech Group (USA) was used to check the interaction between MCL1 and Ub.

### Immunohistochemistry Assay

After the tumor tissues were paraffin sectioned, they were incubated with MCL1 (1:100) FBXW7 (1:100) or Ki67 (1:100) antibodies at 4°C for overnight. Then the paraffin sectioned were incubated with HRP-conjugated secondary antibodies for 3 hours at room temperature. Subsequently, they were stained by DAB, and tissues were counterstained with hematoxylin. Finally, photographs were taken by the inverted microscope.

### The screen of BCL2-drug-resistant-cell-lines

The MKN-45 and SGC-7901 were cultured in 1640 medium with HA14-1(9 μM) for a week. Then the dead cells were washed with PBS. The remaining living cells were diluted in 96-well plate by gradient dilution method and maintained a high concentration of HA14-1. Continue to cultivate for 3 weeks. Finally, the surviving monoclonal cells were selected, and amplification cultured. Finally, the HA14-1-resistant cell lines (MKN-45-R and SGC-901-R) were obtained.

### Autophagy flux detection

The mRFP-GFP-LC3-adenovirus (HB-AP2100001) was purchased from HanBio (Shanghai, China). Subsequently，mRFP-GFP-LC3B-adenovirus was added into the medium of MKN-45 and SGC-7901, cultured for 24h. Then, removed the old medium and added the fresh 1640 medium (with 10 % fetal bovine serum, 1% Penicillin, Streptomycin, and 20 μM LH). After 48h, confocal microscopy was used to record the experimental results.

### Statistical analysis

Statistical analyses were obtained by the Graphpad prism software. Data were showed as mean ± SEM and analyzed by unpaired 2-tailed t-tests. P-values of <0.05 (*), <0.01 (**) and <0.001 (***) were considered statistically significant.

## Results

### Lycorine hydrochloride inhibits gastric cancer cells growth and tumorigenesis

To investigate the effect of LH on gastric cancer cells, we treated gastric cancer cell lines, MKN-45 and SGC-7901, with different concentrations of LH (10, 20 and 40 μM) for 48 h. Dimethylsulfoxide (DMSO) was used as control. MKN-45 and SGC-7901 cells exposed to LH showed significant morphological changes and cell numbers decrease in a dose-dependent manner (Fig. S1A). Cell viability assay was analysed by MTT and BrdU. MTT assay showed that LH significantly inhibited cells growth (Fig. 1A), and its semi-lethal concentration (IC50) was approximately 20 μM. In consideration of the potential toxicities of LH, we chose 20 μM LH as an indicated concentration for further investigations. BrdU assay showed that DNA synthesis decreased after treatment with 20 μM LH for 48 h (Fig. 1B). We further examined the cell cycle to assess whether LH inhibited cell proliferation by causing cell cycle arrest. Flow cytometry analysis showed that LH could block cell cycle progression at S phase (Fig. 1C). Furthermore, the related cyclins were detected by western blotting. We found that LH could significantly inhibit the expression of CDK1 and CDK2 in a dose and time dependent manner (Fig. 1D and Fig. S1C). To further investigate the effects of LH in vivo, SGC-7901 cells were injected subcutaneously into 5-week-old female nude mice. The results demonstrated that mice injected with LH had smaller tumor volume and less tumor weight comparing with the mice injected with DMSO (Fig. 1E, F). Immunohistochemical (IHC) staining with Ki67 further supported the results that LH inhibits tumourigenecity in gastric cancer cells (Fig. 1G). In conclusion, LH could dramatically inhibit the growth and tumorigenesis of gastric cancer cells.

### Lycorine hydrochloride induces gastric cancer cells apoptosis

In addition to inhibiting the proliferation of gastric cancer cells, could LH affects apoptosis or autophagy? The mRFP-GFP-LC3-adenovirus system confirmed that there was no obvious autophagy flux in gastric cancer cells after treatment with LH (Fig. S1D). By flow cytometry apoptotic analysis, we found that LH could significantly induce apoptosis of gastric cancer cells (Fig. 2A). Meanwhile, TUNEL staining was

analysed. Similarly, TUNEL staining showed that LH did induce apoptosis (Fig. 2B). To further confirm these results, we treated MKN-45 and SGC-7901 cells with 10, 20 and 40 μM LH (DMSO was used as control). Cleaved Caspase 3 (C-Caspase 3) and cleaved poly ADP-ribose polymerase (C-PARP), which are apoptosis-related markers, were tested and results showed that C-Caspase 9, C-Caspase 3 and C-PARP increased in a concentration-dependent manner (Fig. 2C). Moreover, C-Caspase 9, C-Caspase 3 and C-PARP from cells treated with LH (20 μM) for different time (0, 12, 24, 48h), were detected. The results also showed that C-Caspase 9, C-Caspase 3 and C-PARP were increased in a time-dependent manner (Fig. 2D).

## Overexpression of MCL1 restores cells proliferation and decreases apoptosis induced by lycorine hydrochloride

According to online database (https://www.cbligand.org/HTDocking/searchstruct.php) prediction, LH may have the possibility of interactions with MCL1 (Fig S3A). As is reported in literature, we knew that MCL1 is highly expressed in gastric cancer, and the prognosis of patients with high expression of MCL1 is worse [32]. After treatment with different concentration of LH (10, 20 and 40 μM, DMSO was used as control) for 48 h, we found that MCL1, a critical anti-apoptotic protein from BCL2 family, was significantly decreased in a dose-dependent manner in both MKN-45 and SGC-7901 cells (Fig. 3A). Moreover, MKN-45 and SGC-7901 cells were treated with 20 μM LH for 0, 12, 24 and 48 h. The results indicated that MCL1 was reduced in a time-dependent manner as well (Fig. 3A). However, BCL2 and BAK did not markedly change with the addition of LH (Fig. S3B). Through lentivirus transfection, stable MKN-45 and SGC-7901 cell lines with exogenous overexpression of MCL1 were obtained. Western blotting results demonstrated that MCL1 was up-regulated after infection with lentivirus. DMSO and empty vector were used as control (Fig. 3B). According to the MTT experiment, we found that the growth rate of overexpressing MCL1 cells after LH treatment was significantly higher than the group of empty vector treated with LH (Fig. 3C). After treatment with 20 μM LH for 48 h, BrdU staining was analysed. Results illustrated that overexpression of MCL1 rescued the DNA synthesis decreased by LH (Fig. S3E). To further explore the effect of MCL1 on cell cycle arrest induced by LH, flow cytometry experiment was performed. The results showed that MCL1 could partly rescue the cell cycle arrest caused by LH (Fig. S3F). Subsequently, western blotting results showed that under the LH treatment, the related cyclins, such as CDK1 and CDK2, were partly restored after overexpression of MCL1 (Fig. S3G).

Meanwhile, the flow cytometry for apoptotic analysis results indicated that overexpression of MCL1 decreased percentage of apoptotic cells treated by LH (Fig. S3C). Besides, the TUNEL staining experiment was carried out, and the results showed that MCL1 could partially rescue apoptosis induced by LH (Fig. S3D). To further confirm the above results, western blotting was performed, and C-PARP and C-Caspase 3 were partially restored in the group of MCL1 overexpression (Fig. 3D). To further explore the mechanism of apoptosis, an immunoprecipitation experiment was carried out, and the results showed that the interaction of MCL1-BIM was decreased, and the interaction of BIM-BAX was raised after treatment with different concentration gradients LH for 48 h (Fig. 3E). In conclusion, the above results showed that MCL1 could obviously restore cell proliferation and decrease apoptosis induced by LH.

### Lycorine hydrochloride decreases the protein stability of MCL1 through FBXW7

To further explore the molecular mechanism of LH regulating MCL1, we performed qRT-PCR experiments. We found that the mRNA level of MCL1 did not decrease after LH treatment, but increased slightly (Fig. 4A). Therefore, we speculated that LH might affect the protein stability of MCL1. Indeed, LH could decrease the turnover rate of MCL1 in the presence of the de novo protein synthesis inhibitor cycloheximide (CHX) (Fig. 4B). Meanwhile, the western blotting analysis revealed that the proteasome inhibitor MG132 could partly rescue down-regulation of MCL1 after treatment with LH. (Fig. 4C). Further, after examining the ubiquitination levels of MCL1, we found that LH increased the ubiquitination levels of MCL1 (Fig. 4D). Therefore, we analyzed the related genes which regulate the ubiquitination level of MCL1 by qRT-PCR. The primers used were listed in Table 1. The results showed that ubiquitin E3 ligase FBXW7 was up-regulated after treatment with LH (Fig. S4A). Furthermore, the western blotting showed that FBXW7 (not HUWEI or β-TrCP) was up-regulated after adding LH (Fig. 4E and Fig. S4B). After down-regulating FBXW7 in the gastric cancer cells, we found that the down-regulation of MCL1 expression induced by LH could be partially restored (Fig. 4F). Finally, we concluded that LH could down-regulate the stability of MCL1 through FBXW7, thereby promoting gastric cancer cells apoptosis, and inhibiting cells growth.

### Lycorine hydrochloride induces apoptosis of BCL2-drug-resistant gastric cancer cell lines

According to multiple lines of evidence indicated, MCL1 is commonly up-regulated in various cancers and is considered as a primary factor to resistance the treatment with the BCL2 inhibitor [33]. Besides, acquired resistance is an obstacle for most of drugs ever used in oncology. Our findings indicated that, with prolonged treatment of HA14-1, sensitive gastric cancer cell lines could spontaneously select to resistant it. So, could LH affect HA14-1-resistant cells? Based on our described screening method, we obtained drug-resistant-cell-lines (MKN-45-R, SGC-7901-R), which were resistant to HA14-1 (Fig. 5A). MCL1 silencing promoted the apoptosis of drug-resistant-cell-lines, and the combination with HA14-1 (BCL2 specific inhibitor) significantly increased the apoptosis (Fig.5B). To further detect the effect of LH on BCL2 resistant cell lines, trypan blue staining, TUNEL staining and western blotting were performed. Trypan blue staining and TUNEL staining showed that LH could also induce apoptosis in drug-resistant-cell-lines (Fig. 5C and Fig. 5D). Subsequently, western blotting showed that LH could also induce the up-regulation of apoptosis-related proteins, C-Caspase 9, C-Caspase 3 and C-PARP in BCL2-drug-resistant gastric cancer cell lines with a dose-time gradient effect (Fig. 5E). In brief, the above experiments showed that LH exactly has therapeutic effect on BCL2-resistant gastric cancer cell lines.

### The combination oflycorine hydrochloride and HA14-1 enhances the therapeutic effect on gastric cancer

According to our research above, the trypan blue staining showed that HA14-1 and LH could both induce apoptosis in gastric cancer cell lines (Fig. 6A). Besides, the apoptosis rate induced by the combination of the two drugs was significantly higher than that sum of the ratio of apoptosis induced by the two separated drugs (Fig. 6A). Similarly, western blotting results showed that the combination of LH and HA14-1 could significantly induce the expression of apoptosis-associated proteins such as C-Caspase 9,

C-Caspase 3, and C-PARP (Fig. 6B). To further investigate the treatment effects of the combination of HA14-1 and LH in vivo, we carried out the PDX model experiment. Tumor masses (GAM-AD) from The Ninth People's Hospital of Chongqing were transplanted subcutaneously into famale nude mice. After two weeks, the mice were randomly divided into 3 groups, then separately injected with LH(30mg/kg), LH (30mg/kg) + HA14-1 (2.5mg/kg) and DMSO once a day. The equivalent amount of DMSO was used as the control. Mice weight was measured every four days. The weight of the mice did not markly differ between the control and drugs treatment groups (Fig. 6D), indicating that the drugs were hypotoxic in mice. However, the tumor weight was significantly decreased in mice. In addition, compared with treatment with LH only, the treatment with LH+HA14-1 have a more obvious therapeutic effect (Fig. 6C). Further, the IHC results showed that the Ki67 staining of LH treated mice was significantly reduced, and the expression of Ki67 was significantly decreased after treatment with LH+HA14-1 compared with treatment with LH only (Fig. 6E). Immunohistochemical (IHC) staining with MCL1 further supported the results that LH inhibited tumourigenecity in gastric cancer through down-regulating the expression of MCL1. In a word, the results indicated that LH combined with HA14-1 exhibited a more significant inhibitory effect than LH alone in vivo.

# Discussion

As one of the most common and deadly cancers in the world, gastric cancer is hard to cure, which is the third leading cause of cancer-related death in men and the fifth leading cause of cancer-related death in women [34]. We focused on the monomer of traditional Chinese medicine. As a tested drug, LH has not only little toxic side effects, but also the advantage of clear molecular formula. Through our study, we found that LH could inhibit the gastric cancer growth and induce apoptosis of gastric cancer cells.

Further, according to the database prediction (Fig S3A), LH may have the possibility of interactions with MCL1. So we focused on MCL1, which is amplified in many types of tumor, such as lung, breast, prostate, pancreatic, ovarian and cervical cancers, melanoma and leukemia, etc [35]. Besides, the MCL1 has recently been regarded as a promising target for cancer treatment [35, 36]. In view of the previous reports, we knew that MCL1 is highly expressed in gastric cancer, and the prognosis of patients with high expression of MCL1 is worse [32]. In this study, LH was proved to cause cell cycle arrest at S phase and induced apoptosis in gastric cancer by inhibiting MCL1, which is consistent with the previous reports that MCL1 is involved in cell cycle by improving the stability of CDK2 protein [37]. Furthermore, we further confirmed that LH did not down-regulate the mRNA level of the MCL1. MCL1 protein is extremely unstable, with a very short half-life [38]. MCL1 degradation is regulated by its phosphorylation at several sites, leading to subsequent ubiquitination by E3 ligases such as F-box and WD repeat domain-containing 7 (FBXW7), HUWEI/Mule, and β-TrCP [39–43]. In addition, the stability of MCL1 protein is also regulated by deubiquitinase such as: JOSD1, DUB3, USP13, USP9X [44–47]. Therefore, we speculated whether LH affected the stability of MCL1 by directly binding, indirectly regulating, or the above two ways. Subsequently, qRT-PCR and western blotting experiments showed that FBXW7 could respond to LH and has a negative correlation with MCL1 expression. After FBXW7 was down-regulated, it could obviously save the down-regulation of MCL1 caused by LH. Hence, we suggested that LH may mainly regulate the

proliferation and apoptosis of gastric cancer by regulating FBXW7-MCL1 axis. Moreover, the direct combination between LH and MCL1 needs further verification.

According to the previous report, overexpression of MCL1 is the cause of drug resistance of several chemotherapeutic agents. For example, overexpression of MCL1 induces resistance to many widely used anti-cancer therapies drugs such as BCL2 inhibitors, such as paclitaxel, vincristine, and gemcitabine [48, 49]. In this study, we screened BCL2-drug-resistant-cell-lines (MKN-45-R and SGC-7901-R). The qRT-PCR assay showed that the transcriptional level of MCL1 in BCL2-drug-resistant gastric cancer cell lines was higher than that in normal gastric cancer cell lines (Fig. S5). Besides, the apoptosis of the drug-resistant gastric cancer cell lines could also be induced by down-regulating MCL1 or adding LH. These results confirmed that LH not only induces apoptosis of gastric cancer but also may be a potential therapeutic drug for patients with BCL2-drugs- resistance. There is no doubt that BCL2 and MCL1, two anti-apoptotic members of the BCL2 family, have similar structures. The down-regulation of the BCL2 or MCL1 could counteract the apoptosis by up-regulation of the other one. In our study, we found that the combination of BCL2 and MCL1 inhibitors could induce gastric cancer cells more significantly apoptosis. The combination of LH and HA14-1 induced remarkable tumor growth inhibition in our PDX model.

# Conclusions

Taken together, in this study, lycorine hydrochloride (LH), an extract of *Lycoris radiate*, was proved to reduce MCL1 protein stability by FBXW7, causing the apoptosis of gastric cancer cells. Besides, as a potential inhibitor of MCL1, LH could kill the BCL2-drug-resistant-cell-lines. Meanwhile, PDX model experiment showed that LH combined with HA14-1 greatly inhibited the growth of gastric cancer in vivo. In sum, the above results showed that LH is worthy being further researched as a clinical drug for gastric cancer treatment, and these findings provided some information for the exploration of MCL1 inhibitors.

# Declarations


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## Authors' contributions

Chaowei Deng and Guangzhao Pan conducted the animal experiment. Kui Zhang, Xue Wang and Zhen Dong revised the article. Gaichao Zhao, Mengqin Tan, Xiaosong Hu and Shaomin Shi carried out molecular/cell biology experiments. Juan Du, Haoyan Ji and Xiaowen Wang performed statistical analyses. Chongyang Li designed the study, and wrote the manuscript. Hongjuan Cui and Liqun Yang supervised the study and revised the manuscript.

## Authors' information

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### Availability of data and materials

All the data reported by the manuscript are publicly available and the materials are also freely available [50].

### Ethics approval and consent to participate

All animal experiments were conducted in accordance with the Guidelines for Animal Health and Use (Ministry of Science and Technology of China, 2006). The tumor mass of GAM-AD was from The Ninth People's Hospital of Chongqing. With the consent of the patient's family, we carried out the PDX model experiment. Besides, the patient's privacy has been fully protected.

### Consent for publication

The corresponding author and all the co-authors have agreed to the publication of the manuscript to Journal of Experimental and Clinical Cancer Research as a research article and declare that they have no conflict of interest as to the results presented.

### Competing interests

The authors declare no conflict of interest.

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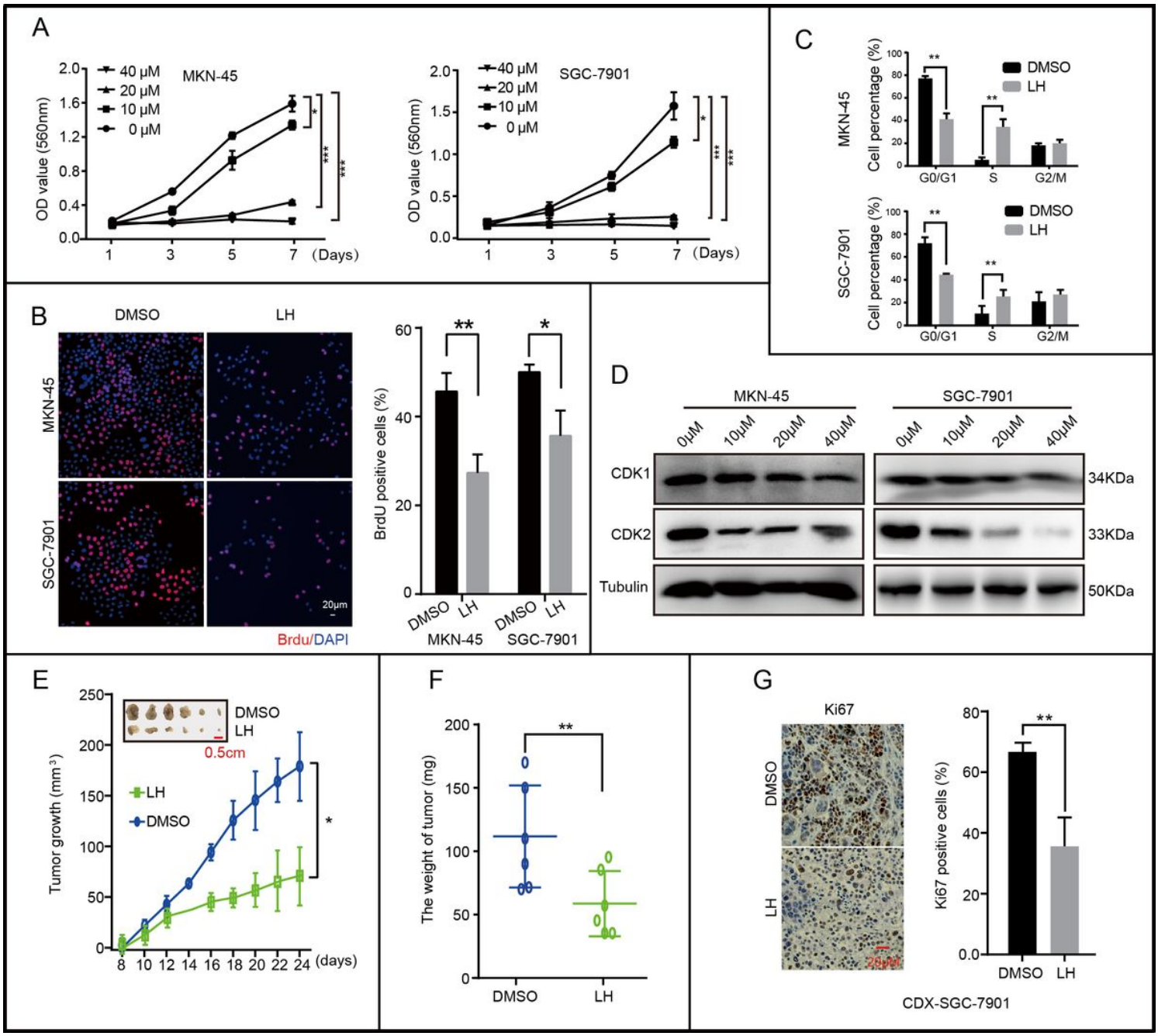
# Table

Table 1 Primers list

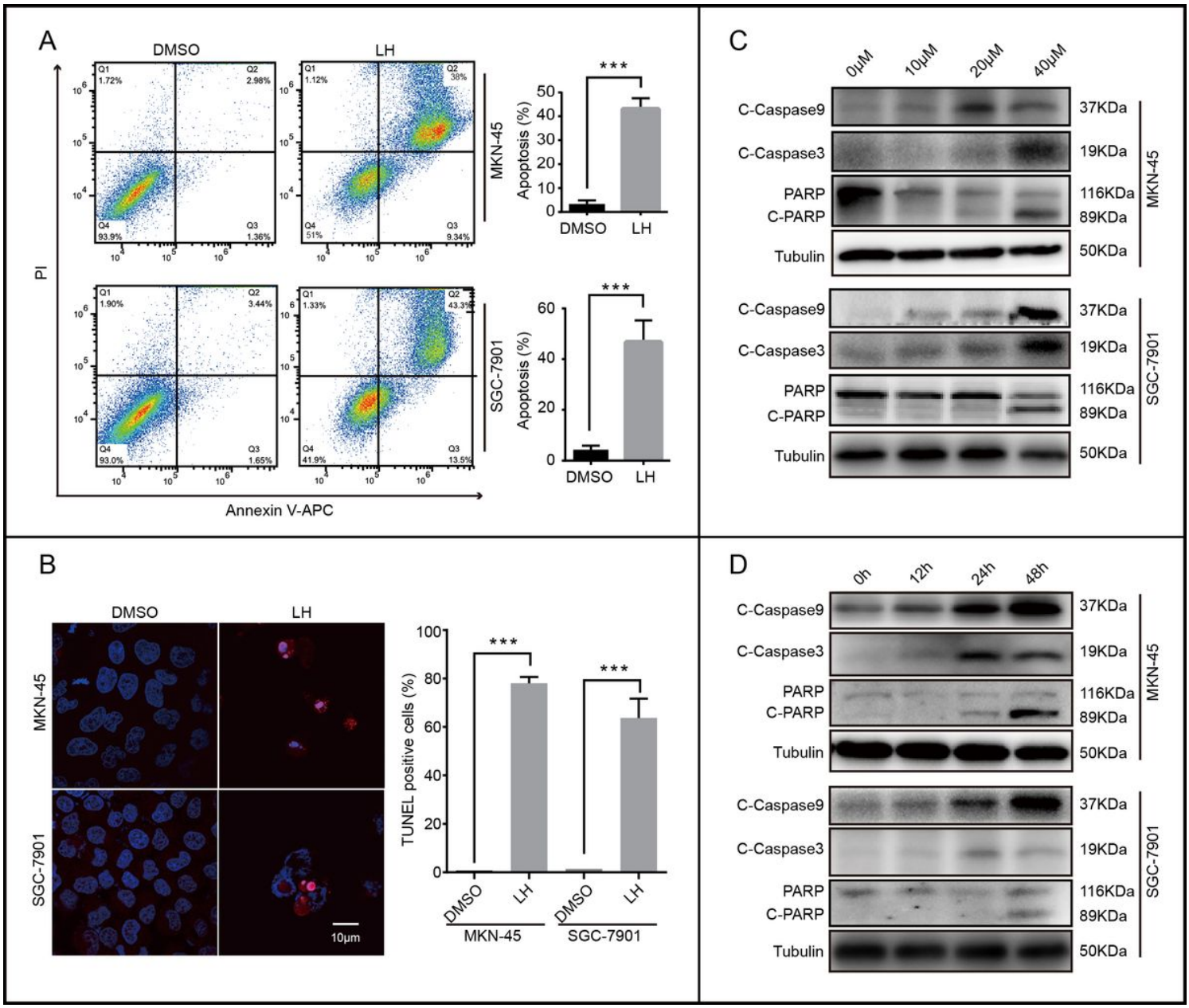
| | |
|---|---|
| qRT-DUB3-F (5’-3’) : | CTATCATTGCGGTCTTTGTCTCC |
| qRT-DUB3-R (5’-3’) : | AAGTGATGCTACAGGCAGTGA |
| qRT-JOSD1-F (5’-3’) : | GGGATACGCTGCAAGAGATTT |
| qRT-JOSD1-R (5’-3’) : | CCATGACGTTAGTGAGGGCA |
| qRT-HUWEI-F (5’-3’) : | TTGGACCGCTTCGATGGAATA |
| qRT-HUWEI-R (5’-3’) : | TGAAGTTCAACACAGCCAAGAG |
| qRT-β-TrCP-F (5’-3’) : | CCAGACTCTGCTTAAACCAAGAA |
| qRT-β-TrCP-R (5’-3’) : | GGGCACAATCATACTGGAAGTG |
| qRT-FBXW7-F (5’-3’) : | GGCCAAAATGATTCCCAGCAA |
| qRT-FBXW7-R (5’-3’) : | ACTGGAGTTCGTGACACTGTTA |
| qRT-USP13-F (5’-3’) : | TCTCCTACGACTCTCCCAATTC |
| qRT-USP13-R (5’-3’) : | TCTCCTACGACTCTCCCAATTC |
| qRT-USP9X-F (5’-3’) : | TCGGAGGGAATGACAACCAG |
| qRT-USP9X-R (5’-3’) : | GGAGTTGCCGGGGAATTTTCA |
| qRT-GAPDH-F (5’-3’) : | GGAGCGAGATCCCTCCAAAAT |
| qRT-GAPDH-R (5’-3’) : | GGCTGTTGTCATACTTCTCATGG |
| shMCL1#1-F1 (5’-3’) : | CCGGCGCCATCATGTCGCCCGAAGACTCGAGTCTTCGGGCGACATGATGGCGTTTTTG |
| shMCL1#1-R1 (5’-3’) : | AATTCAAAAACGCCATCATGTCGCCCGAAGACTCGAGTCTTCGGGCGACATGATGGCG |
| shMCL1#2-F2 (5’-3’) : | CCGGCTTCCATGTAGAGGACCTAGACTCGAGTCTAGGTCCTCTACATGGAAGTTTTTG |
| shMCL1#2-R2 (5’-3’) : | AATTCAAAAACTTCCATGTAGAGGACCTAGACTCGAGTCTAGGTCCTCTACATGGAAG |

# Figures



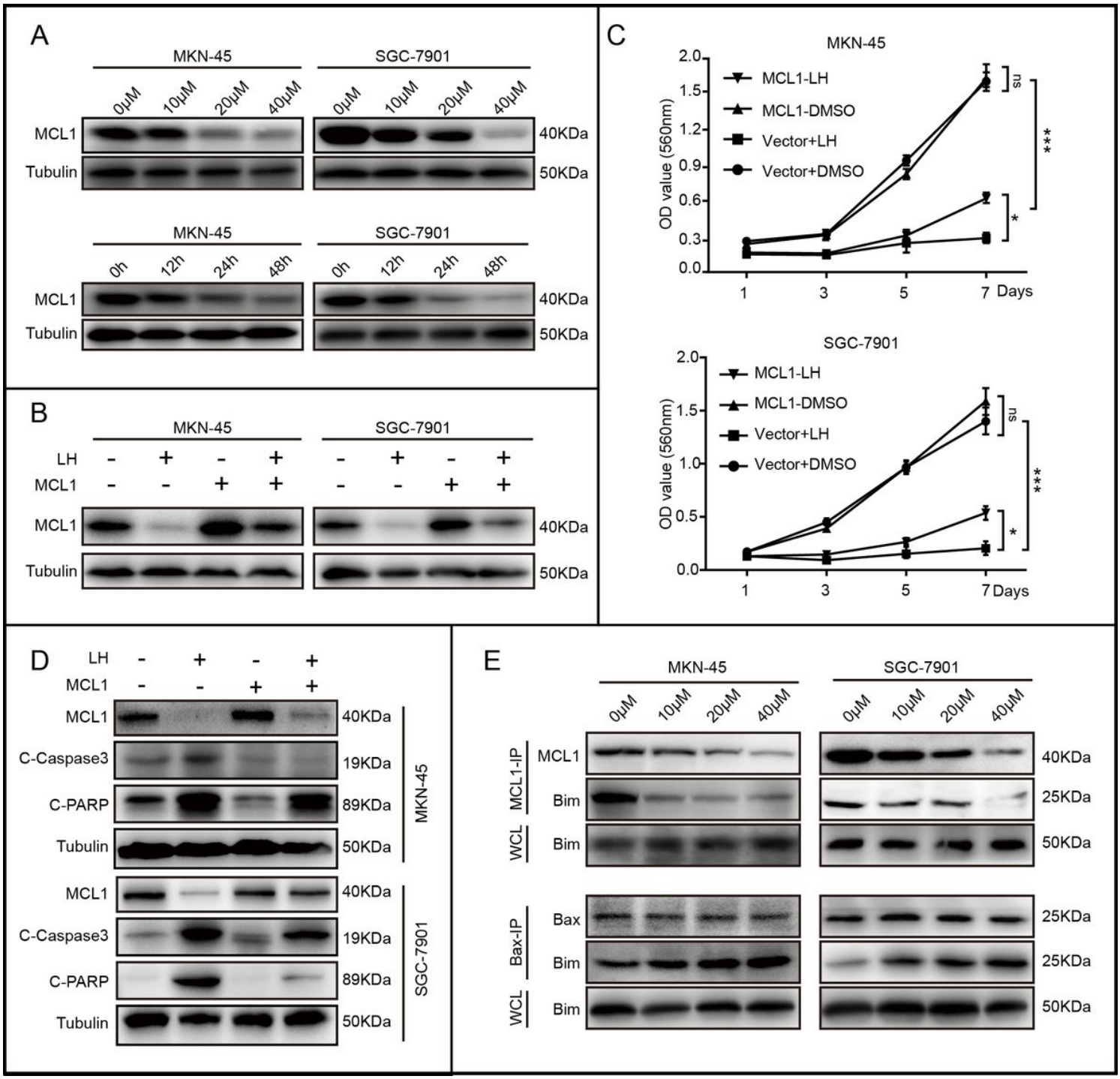

## Figure 1

Lycorine hydrochloride inhibits gastric cancer cells growth and tumorigenesis. (A) Viability of MKN-45 and SGC-7901 cells after treatment with 10, 20, and 40 μM LH. DMSO was used as control. (B) BrdU-positive MKN-45 and SGC-7901 cells after treatment with 20 μM LH for 48h. DMSO was used as control. The histogram demonstrated the results of the quantification of the number of BrdU-positive cells in MKN-45 and SGC-7901 cells. (C) Cell cycle of MKN-45 and SGC-7901 cells treated with 20 μM LH for 48h were analyzed by flow cytometry. DMSO was used as control. Percentage indicated MKN-45 and SGC-7901 cells at different phase. (D) The expression of CDK1 and CDK2 in gastric cancer cells treated with different concentration of LH (0, 10□20, 40 μM) for 48h. Tubulin was used as internal reference. All data were analyzed by unpaired Student's t-tests and were showed as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.




## Figure 2

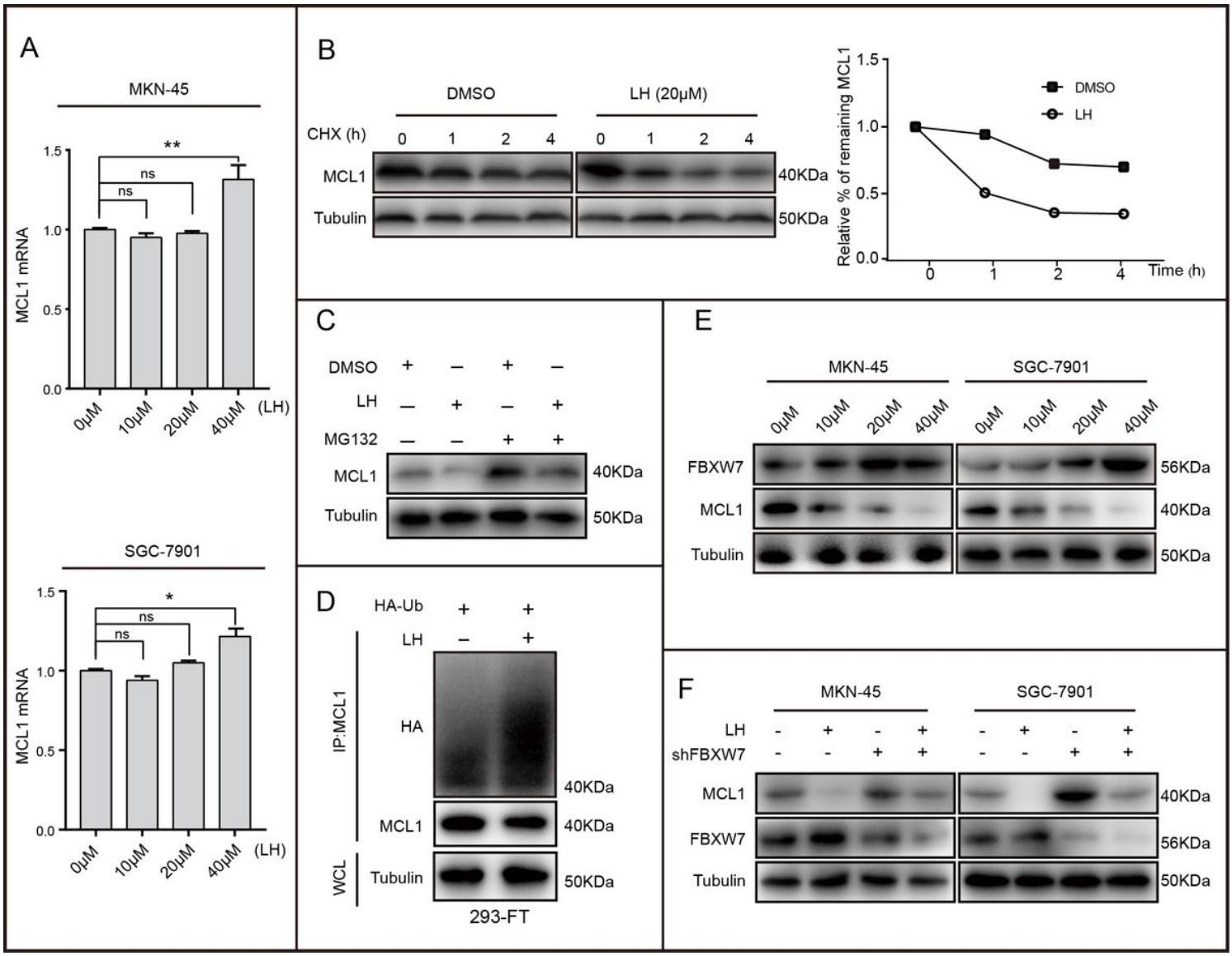
Lycorine hydrochloride induces apoptosis in gastric cancer cells. (A, B) Apoptosis of MKN-45 and SGC-7901 cells treated with 20 μM LH for 48h were examined by flow cytometry and TUNEL staining. DMSO was used as control. (C, D) The expression of apoptotic protein, including C-Caspase 9, C-Caspase 3, PARP and cleaved PARP in gastric cancer cells treated with LH at different concentrations and time gradients. DMSO was used as control. Tubulin was used as internal reference. All data were analyzed by unpaired Student's t-tests and were showed as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.




## Figure 3

Overexpression MCL1 restores cell proliferation and decreases apoptosis induced by lycorine hydrochloride. (A) The expression of MCL1 in MKN-45 and SGC-7901 cells treated with different concentration LH (10, 20 and 40 μM) for 48h and treated with 20 μM LH for different time (0, 12, 24 and 48 h). DMSO was used as control. Tubulin was used as internal reference. (B) The expression of MCL1 in 20 μM LH-treated cells overexpressing MCL1 or empty vector. (C) Growth curve of MKN-45 and SGC-7901 cells overexpressing MCL1 after treatment with 20 μM LH. DMSO and empty vector were used as control. (D) The expression of MCL1, cleaved-PARP and C-Caspase3 were checked in MKN-45 and SGC-7901 cells

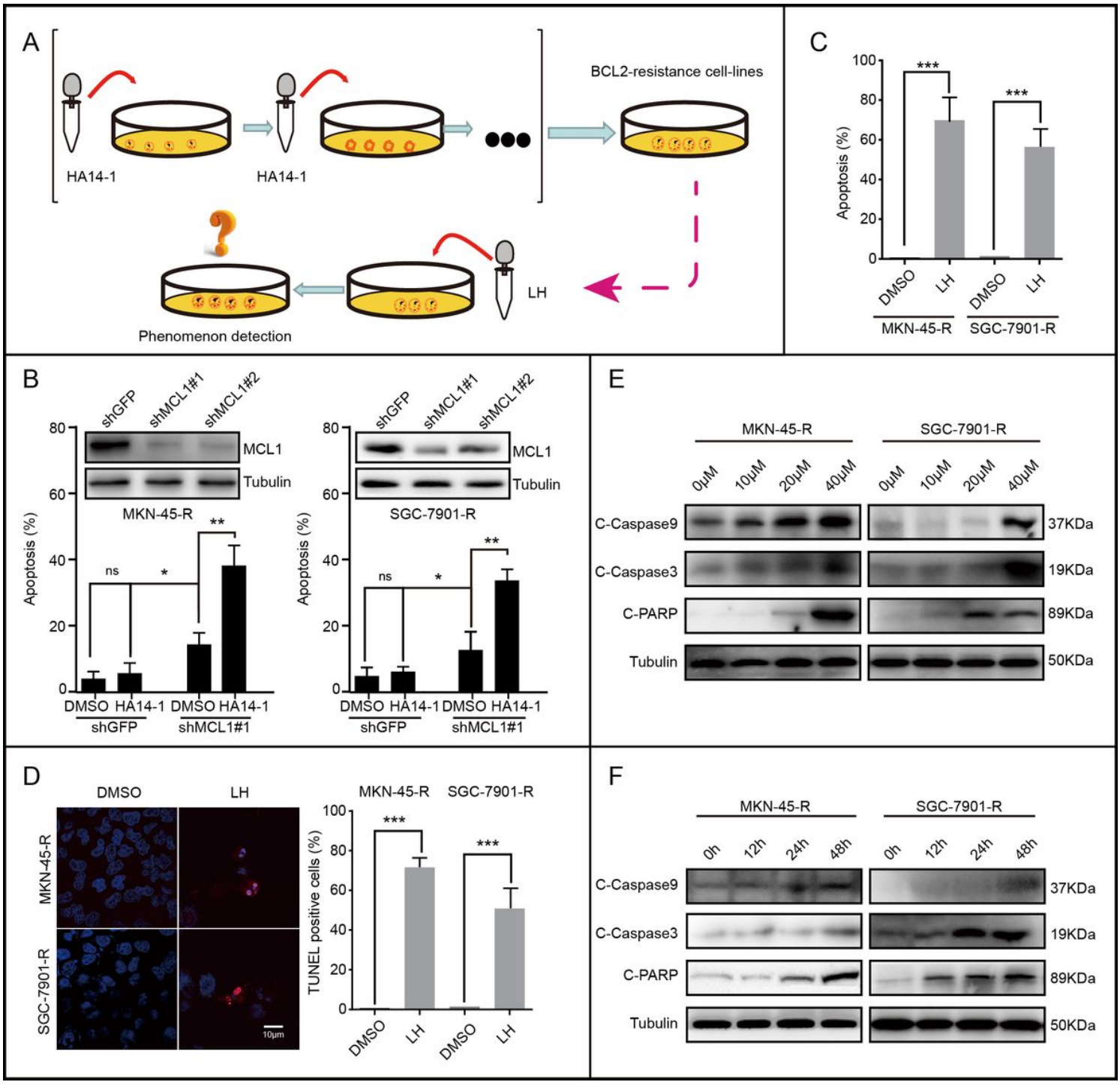
overexpressing with MCL1 after treatment with 20 μM LH for 48 h. DMSO and empty vector were used as control. Tubulin was used as internal reference. (E) The interaction of MCL1 and BIM; BIM and BAX were detected after treating with different concentration LH (10, 20 and 40 μM) for 48 h by immunoprecipitation. DMSO was used as control. All data were analyzed by unpaired Student's t-tests and were showed as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.




## Figure 4

Lycorine hydrochloride affects the stability of MCL1 protein through FBXW7. (A) Quantitative PCR was performed to detect the mRNA level of MCL1 in gastric cancer cells after treatment with LH. (B) 293-FT cells were treated with LH (20 μM) or DMSO and were then treated with CHX (100 μg/ml) for the indicated times. Cell lysate was immunoblotted with the indicated antibodies. The density of MCL1 was measured, and the integrated optical density (IOD) was measured. The turnover of MCL1 was indicated graphically. (C) Cell lysate was prepared from 293-FT cells treated with DMSO or LH that had been treated with or without MG132 for 8 h. Equal amounts of cell lysate was immunoblotted with the indicated antibodies. (D) The ubiquitination of MCL1 in 293-FT cells was enhanced by treatment with LH. (E) Western blotting

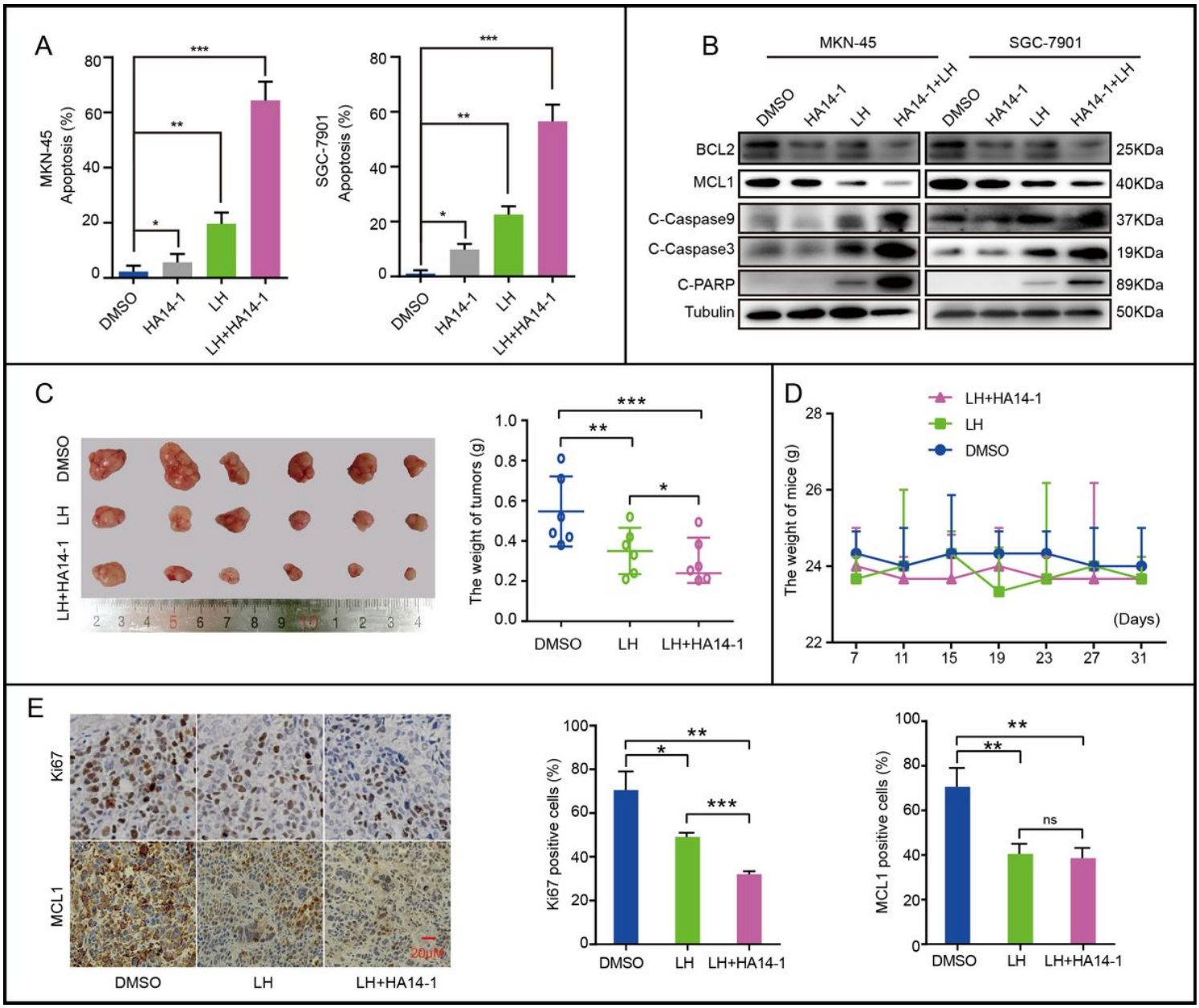
assays were performed to detect the expression of FBXW7 and MCL1 in MKN-45, SGC-7901 cells after treatment with LH. Tubulin was as internal reference. (F) Protein expression levels of MCL1 and FBXW7 were analyzed by western blotting in MKN-45, SGC-7901 cells. All data were analyzed by unpaired Student's t-tests and were showed as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.




## Figure 5

Lycorine hydrochloride induces apoptosis of BCL2-drug-resistant gastric cancer cell lines. (A) Screening of HA14-1 resistant cell lines (Detailed screening methods was described in the materials and methods). (B) Western blotting analysis of MCL1 expression in MKN-45-R and SGC-7901-R cell lines expressing

shGFP, shMCL1#2, shMCL1#4. Trypan blue staining was used to analyze the apoptosis induced by DMSO or HA14-1 in MKN-45-R and SGC-7901-R cell lines expressing shGFP, shMCL1#1. (C, D) Apoptosis was analyzed in MKN-45-R, SGC-7901-R cells after treatment with 20 μM LH for 48 h by trypan blue and TUNEL staining. Apoptotic rate of MKN-45-R, SGC-7901-R cells in panel was quantified. (E, F) The expression of apoptotic protein, including C-Caspase 9, C-Caspase 3, PARP and C-PARP in BCL2-drug-resistant gastric cancer cells treated with LH at different concentrations and time gradients. DMSO was used as control. Tubulin was used as internal reference. All data were analyzed by unpaired Student's t-tests and were showed as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.




## Figure 6

The combination of lycorine hydrochloride and HA14-1 enhances the therapeutic effect on gastric cancer. (A) Apoptosis of MKN-45 and SGC-7901 cells treated with LH (20 μM) , HA14-1(9 μM) or LH (20 μM) + HA14-1(9 μM) for 48 h were examined by trypan blue staining. DMSO was used as control. Apoptotic rate

of MKN-45 and SGC-7901 cells was quantified. (B) Western blotting was used to detect the expression of apoptotic protein, including BCL2, MCL1, C-Caspase 9, C-Caspase 3, PARP and C-PARP in MKN-45 and SGC-7901 cells after 48 hours of treatment with LH (20 μM), HA14-1 (9 μM) or LH (20 μM) + HA14-1 (9 μM). DMSO was used as control. (C) Tumor weight of indicated mice. DMSO was used as control. (D) The weight of the mice treated with DMSO, LH or LH + HA14-1 was measured. (E) IHC of MCL1 and Ki67 in indicated tumors. Scale bar =20 μm. Gray value of IHC positive signal in panel was quantified. All data were analyzed by unpaired Student's t-tests and are shown as the means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

## Supplementary Files

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