Lycorine inhibits the growth and metastasis of breast cancer through the blockage of STAT3 signaling pathway

Jian Wang*, Jie Xu, and Guoqiang Xing
Department of General Surgery, The Fifth Central Hospital of Tianjin, Tianjin 300450, China

*Correspondence address. Tel/Fax:+86-22-65665880; E-mail: 13820620690@163.com

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Abstract
Signal transducer and activator of transcription 3 (STAT3) is involved in the growth and metastasis of breast cancer, and represents a potential target for developing new anti-tumor drugs. The purpose of this study is to investigate whether Lycorine, a pyrrolo[de]phenanthridine ring-type alkaloid extracted from Amaryllidaceae genera, could inhibit breast cancer by targeting STAT3 signaling pathway. The human breast cancer cell lines were incubated with various concentrations of Lycorine, and cell proliferation, colony formation, cell cycle distribution, apoptosis, migration and invasion were assayed by several in vitro approaches. Results showed that Lycorine significantly suppressed cell proliferation, colony formation, cell cycle distribution, apoptosis, migration and invasion, as well as induced cell apoptosis, but showed no apparent impact on cell cycle. In addition, the effect of Lycorine on tumor growth and metastasis in nude mouse models was investigated, and results showed that Lycorine significantly inhibited tumor growth and metastasis in vivo. Mechanistically, Lycorine significantly inhibited STAT3 phosphorylation and transcriptional activity through upregulating SHP-1 expression. Lycorine also downregulated the expressions of STAT3 target genes, including Mcl-1, Bcl-xL, MMP-2, MMP-9, which are involved in apoptosis and invasion of breast cancer. Taken together, these findings suggest that Lycorine may be a promising candidate for the prevention and treatment of human breast cancer.

Key words: breast cancer, Lycorine, STAT3, tumor growth, metastasis

Introduction
Breast cancer is one of the most common malignant tumors affecting women worldwide [1]. Although there are various therapies available for breast cancer, the mortality and recurrence rate are still high [2]. Moreover, the current therapies that are efficacious against breast cancer also have undesirable side effects [3,4]. Therefore, new candidates for breast cancer that possess potential anti-tumor activity and low toxicity are urgently needed.

Signal transducer and activator of transcription 3 (STAT3) is a critical point of convergence for several signaling pathways in cancer cells [5]. Once cytokines, such as interleukin-6 (IL-6), bind to their cognate receptors, tyrosine kinases, including Janus-like kinases (JAKs) and c-Src kinase, are activated and proceed to phosphorylate and activate STAT3 [6]. Upon activation, STAT3 undergoes phosphorylation-induced homodimerization, leading to nuclear translocation, DNA binding, and transcription of target genes [7]. Because some of these gene products are key proteins involved in the regulation of tumor progression, STAT3 has emerged as a potential anti-cancer target in various malignant tumors [8,9]. STAT3 has also been reported to be overexpressed and constitutively activated in breast cancer, and its constitutive activation contributes to the progression of breast cancer [10–12]. Therefore, STAT3 may be a promising therapy target for breast cancer.

Lycorine, a pyrrolo[de]phenanthridine ring-type alkaloid extracted from Amaryllidaceae genera, and was reported to possess various effects, such as, anti-viral, anti-malarial, and anti-inflammatory effects.
activities [13–15]. Recent research has focused on the potential anti-cancer activity of Lycorine. It has been reported that Lycorine suppresses the progression of various malignant tumors, such as leukemia, cervical cancer, multiple myeloma, and prostate cancer [16–22]. However, the exact role of Lycorine in the progression of breast cancer is still unclear. The purpose of present study is to detect the effects of Lycorine on the growth and metastasis of breast cancer in vitro and in vivo.

Materials and Methods

Cell lines, reagents, and antibodies
The MDA-MB-231, MCF-7 and HMEC cell lines were obtained from the American Type Culture Collection (Manassas, USA). All cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum. Z-VAD-FMK, IL-6 and AG490 were purchased from Sigma-Aldrich (St Louis, USA). Small-interference RNA SHP-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Primary antibodies against Bcl-xl, Mcl-1, cleaved-caspase-3, cleaved-caspase-9, cleaved-PARP, p-STAT3, and STAT3 were purchased from Cell Signaling Technology (Danvers, USA). Anti-GAPDH antibody and secondary antibodies against mouse IgG-horseradish peroxidase (HRP) and rabbit IgG-HRP were obtained from Santa Cruz Biotechnology. Antibodies against Ki-67, MMP-9, and MMP-2 were obtained from Abcam (Cambridge, USA).

Cell viability assays
Cell viability was determined using CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instruction. MDA-MB-231, MCF-7, and HMEC cells (2.5 × 10³) were seeded into wells of 96-well plate and cultured in 150 μl of medium supplemented with 10% FBS. After 24 h, Lycorine (0–80 μM) was added into the culture medium. After the cells were incubated at 37°C for different time (24 or 48 h), the medium was exchanged for 100 μl of medium and 10 μl of CCK-8 reagent. The cells were incubated for another 2 h at 37°C. Finally, the optical density was measured using an EnSpire™ 2300 Multilabel Reader (PerkinElmer, Waltham, USA) at 450 nm.

Colony formation assays
To determine long-term effects, breast cancer cells were cultured at 10,000 cells/well in a 6-well plate with DMEM. The cells were then exposed to different concentrations of Lycorine for 24 h. After being rinsed with fresh DMEM, cells were allowed to grow for 14 days until the colonies were visible. Then, cells were fixed, stained with 2% crystal violet in ethanol, and photographed.

Cell cycle assays
Cell Cycle was determined by measuring the percentage of cells at G0/G1, S and G2/M phases with a CycleTEST™ PLUS DNA reagent kit (BD Biosciences, Franklin Lakes, USA). Briefly, cells were treated with 0 or 50 μM of Lycorine for 48 h and then incubated with the reagents according to the manufacturer’s instruction. The distribution of the cell cycle phases was detected with a BD FACScan flow cytometer.

Cell apoptosis assays
To measure the rate of cell apoptosis, a PI/Annexin V-FITC apoptosis detection kit (BD Biosciences) was used. Cells were incubated with increasing doses of Lycorine (0, 25 or 50 μM) for 48 h, and then cells were harvested and incubated with Annexin V and PI according to the manufacturer’s instruction. The apoptosis rate was determined by flow cytometry.

Migration and invasion assays
The inhibition of cell migration by Lycorine was examined by wound-healing assay. Briefly, breast cancer cells were allowed to grow into full confluence in 6-well plates, and then the ‘wounds’ were created by a sterile pipette tip. Fresh medium containing 10% FBS and 20 μM of Lycorine was added. Images were taken at 0, 24, or 48 h at 10x on a Nikon Eclipse TS100 microscope (Tokyo, Japan). The invasion assays were performed using Transwell/Boyden chambers coated with Matrigel (Millipore, Billerica, USA). Briefly, 4–5 × 10⁴ cells per well in 250 μl of serum-free medium were pretreated with Lycorine and added to the upper chamber, while 700 μl of medium with 10% FBS was added to the lower chamber. The cells were incubated for 48 h. Then, the non-invaded cells were removed from the membrane of the top compartment with a cotton swab, and the cells that had invaded through the membrane were fixed and stained in 4% paraformaldehyde and 0.01% crystal violet solution. The number of cells that had migrated was determined from six random high-power fields (HPFs) visualized at 100 × magnification, and means were obtained for statistical analysis.

Western blot analysis
Whole cell lysates were prepared using a lysis buffer (20 mM HEPES, pH 7.6, 350 mM NaCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP40, 50 mM NaF, 0.1 mM DTT, 0.1 mM PMSF, and protease inhibitor cocktail) on ice. The lysates were centrifuged at 15,000 g for 10 min. The protein concentration was determined by the Bradford reagent (Bio-Rad, Hercules, USA). Equal amounts of protein were separated on 8%–12% SDS-PAGE and semi-dry transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk, incubated with the respective antibodies overnight at 4°C, and incubated with HRP-conjugated secondary antibody for 1 h. Final detection was performed using enhanced chemiluminescence solution.

DNA-binding assays
Breast cancer cells at 60%–80% confluence were treated with 0, 20, or 40 μM of Lycorine in the presence of 10% FBS for 24 h. A Nuclear extract kit (Clontech, Inc., Mountain View, USA) was used to obtain nuclear extracts. Then, DNA-binding assays were performed by using TransAM STAT3 transcription factor assay kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instruction.

Phosphatase activity assays
The RediPlate 96 EnzChek tyrosine phosphatase assay kit (Molecular Probes, Carlsbad, USA) was used for SHP-1 activity assay. Briefly, protein extracts of breast cancer cells were incubated with anti-SHP-1 antibody in immunoprecipitation buffer overnight. Protein G-Sepharose 4 Fast Flow (GE Healthcare, Piscataway, USA)
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was added to each sample followed by incubation for 3 h at 4°C with rotation and then assayed for phosphatase activity.

Transfection with SHP-1 siRNA
Cells were seeded into 6-well plate and allowed to adhere overnight. Then cells were transiently transfected with siRNA (final concentration, 100 nM) in 6-well plates using the lipid-mediated transfection with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer’s instruction. After 48 h of incubation, the medium was replaced and the cells were incubated with Lycorine or IL-6 for subsequent experiments.

Establishment of stable MDA-MB-231-luciferase cell line
Lentiviral vectors encoding the human firefly Luciferase gene were constructed by GeneChem Corporation (Shanghai, China). The empty vector was used as a negative control. The lentiviral vectors were transfected into MDA-MB-231 cells with a multiplicity of infection (MOI) of 60 in the presence of polybrene (5 μg/ml). About 48 h after transfection, transfected cells were selected for 2 weeks with 2.5 μg/ml puromycin (Sigma). Cells which were obtained 2 weeks after drug selection without subcloning were used for further experiments.

Animal experiment
Six-week-old female athymic nude mice were obtained from the Laboratory Animal Center at the Shanghai University of Traditional Chinese Medicine (Shanghai, China) and were housed in a pathogen-free condition throughout the experimental duration. All procedures and care administered to the animals were approved by the Institutional Ethics Committee of the Fifth Central Hospital of Tianjin. We used six mice in each group to establish subcutaneous xenograft model of breast cancer. Briefly, 2.5 × 10^6 MDA-MB-231 cells were subcutaneously injected into the flanks of nude mice. After the tumors reached about 100 mm^3, mice were divided into 2 groups and received i.p injection either with DMSO or Lycorine (10 mg/kg/day per mouse) every day which lasted for 18 days. During the administration of Lycorine, body weights and the tumor sizes of breast cancer were monitored every 3 days. Mice were continually observed until they were sacrificed. For the lung metastasis model, MDA-MB-231 cells (2 × 10^6) were injected into the nude mice (6/group) through the tail vein. Animals were randomized to receive either Lycorine (10 mg/kg/day per mouse) or DMSO at 1 week after injection. Tumor metastasis were imaged and quantified by bioluminescence every 2 weeks. After 6 weeks, the mice were sacrificed, and their lungs were collected for further experiment.

Immunohistochemistry analysis
Paraffin-embedded tissue sections (4 μm) of breast cancer on poly-lysine-coated slides were deparaffinized and rinsed with 10 mM Tris–HCl (pH 7.4) and 150 mM sodium chloride. Peroxidase was quenched with methanol and 3% hydrogen peroxide. Slides were then placed in 10 mM citrate buffer (pH 6.0) and heated at 100°C for 20 min in a pressurized heating chamber. After incubation with anti-Ki-67 antibody (1:100 dilution) for 1 h at room temperature, slides were thoroughly washed three times with PBS. Bound antibodies were detected using the EnVision Detection Systems Peroxidase/DAB, Rabbit/Mouse kit (Dako, Glostrup, Denmark). The slides were then counterstained with hematoxylin. Images were obtained with a Leica microscope (Leica, Wetzlar, Germany). The results were analyzed using Image-Pro Plus 6.0 software.

Statistical analysis
Data were shown as the mean ± SD of three independent experiments done in triplicate. Statistical analysis between two samples was performed using Student’s t-test. Statistical comparisons of more than two groups were performed using one-way analysis of variance (ANOVA). P < 0.05 was considered as significantly different.

Results
Lycorine inhibits proliferation and colony formation of breast cancer cells
CCK-8 assays were performed to detect the anti-proliferative effects of Lycorine on breast cancer cells. Breast cancer cells (MCF-7 and MDA-MB-231) were cultured in different concentrations (0–80 μM) for 24 h, and then cell viabilities were measured. Results showed that Lycorine suppressed proliferation of breast cancer cells in a dose-dependent manner (Fig. 1A). Breast cancer cells were also treated with AG490 as an additional control; the effects of this treatment on cell viability were shown in Supplementary Fig. S1. Extending drug exposure time to 48 h resulted in additional cytotoxicity, demonstrating that Lycorine also inhibited proliferation of breast cancer cells in a time-dependent manner (Fig. 1B). Lycorine treatment showed lesser toxicity to human mammary epithelial cells (HMECs), which indicated that cells with high levels of constitutive STAT3 activity are more sensitive to Lycorine (Fig. 1A,B). Colony formation assays were performed to investigate the long-term effects of Lycorine on the growth of breast cancer cells. As shown in Fig. 1C,D, the colony formation abilities of breast cancer cells were significantly suppressed by Lycorine in a dose-dependent manner.

Lycorine induces apoptosis in a caspase-dependent manner in breast cancer cells
To explore the specific mechanisms of anti-proliferation effect induced by Lycorine, cell cycle distribution assays were performed to investigate the effects of Lycorine on cell cycle progression. Lycorine showed little impact on the distribution of the cell cycle of breast cancer cells (Supplementary Fig. S2). To further investigate whether anti-proliferation effect induced by Lycorine involved apoptosis, flow cytometry analyses with Annexin V–PI staining were performed. Lycorine-induced apoptosis of MCF-7 and MDA-MB-231 cells in a dose-dependent manner after treatment with Lycorine for 48 h (Fig. 2A). To explore the signaling cascade which mediates Lycorine-induced apoptosis, key proteins of caspase pathway were investigated by western blot analysis. Results showed that Lycorine upregulated the expressions of cleaved-caspase-9, cleaved-caspase-3, and cleaved-PARP in a dose-dependent manner (Fig. 2B). To investigate the dependence of Lycorine-induced apoptosis in the caspase pathway, breast cancer cells were pretreated with a pan-caspase inhibitor, Z-VAD-FMK (10 mM), before treatment with Lycorine. Results showed that pretreatment with Z-VAD-FMK partially decreased Lycorine-induced apoptosis (Fig. 2C). Then, western blot analysis was performed to explore whether Z-VAD-FMK inhibited Lycorine-induced activation of caspase-3. Results showed that Lycorine-induced activation of caspase-3 was partially reversed by pretreatment with Z-VAD-FMK (Fig. 2D). These data suggest that
Lycorine inhibits growth of breast cancer cells by inducing apoptosis in a caspase-dependent manner.

Lycorine suppresses tumor growth of breast cancer in vivo
MDA-MB-231 cells were injected into the flanks of nude mice to establish subcutaneous tumor xenograft model. Mice were divided into 2 groups (n = 6) and treated with Lycorine at 10 mg/kg/day or DMSO. On day 18, mice were sacrificed and the tumor weights were measured. It was found that tumor weights were significantly lower in the Lycorine-treated group compared with the control group (Fig. 3A,B). Otherwise, tumor volumes were also calculated at different time points. Results showed that tumor volumes in the Lycorine-treated group were significantly decreased, compared with the control group (Fig. 3C). However, no marked changes were observed in body, liver and spleen weights in the mice (Fig. 3D–F). Then, immunohistochemical assays were performed to detect the
expression of Ki-67, which is identified as a marker of proliferation. Results showed that Lycorine significantly decreased the number of Ki-67-positive tumor cells, compared with the control group (Fig. 3G,H).

Lycorine suppresses migration and invasion of breast cancer in vitro and in vivo
To investigate the effects of Lycorine on cell motilities, MCF-7 and MDA-MB-231 cells were treated with 20 μM of Lycorine, which showed little effects on cell viability. Results from wound-healing assays showed that Lycorine significantly downregulated cell migration abilities of MCF-7 and MDA-MB-231 cells (Fig. 4A–C). Then, Matrigel-coated Transwell assays were performed to explore the effects of Lycorine on the invasion abilities. Results demonstrated that the invasion abilities of MCF-7 and MDA-MB-231 cells were significantly inhibited after Lycorine treatment (Fig. 4D–F). To further explore the role of Lycorine in the metastasis of breast cancer in vivo, Luciferase-expressing MDA-MB-231 cells were injected through the tail vein of nude mice and tumor metastasis was monitored by bioluminescence imaging. Results showed that stronger illumination signals were detected in control group than in the Lycorine-treated group (Fig. 4G). At the end of treatment, the mice were sacrificed and lungs were excised to perform hematoxylin and eosin staining. Results showed that Lycorine-treated group had less and smaller lung metastases, compared with the control group (Fig. 4H).

Lycorine suppresses constitutive STAT3 phosphorylation through enhancing SHP-1 expression, and reducing the expressions of apoptosis- and invasion-related proteins in breast cancer cells
Previous study has reported that Lycorine inhibits the growth and metastasis of hormone-refractory prostate cancer partly through the blockage of STAT3 signal pathway [22]. Therefore, to elucidate the mechanism by which Lycorine suppresses the growth and metastasis of breast cancer, the effects of Lycorine on STAT3 and STAT3-regulated gene products, including anti-apoptotic proteins (Bcl-xL and Mcl-1) and invasion-related proteins (MMP-2 and MMP-9) were investigated. Results showed that Lycorine reduced the expressions of p-STAT3 (Y705) as well as Bcl-xL, Mcl-1, MMP-2, and MMP-9 in a dose-dependent manner in breast cancer cells without change in total protein levels of STAT3 (Fig. 5A). Then an ELISA-based TransAM STAT3 assay kit was used to explore whether Lycorine affected the transcription activity of STAT3 in breast cancer cells. Results showed that Lycorine eliminated the DNA-binding activities of STAT3 in MCF-7 and MDA-MB-231 cells (Fig. 5B). In addition, Lycorine also inhibited IL-6-induced STAT3 activation in HMEC cells (Supplementary Fig. S3). To further reveal the critical
regulators of Lycorine-induced dephosphorylation of STAT3, the protein expressions of positive STAT3 regulators (JAK2 and p-JAK2) and negative STAT3 regulators (SHP1, SHP-2, and PTP-1B) were detected. Results showed that Lycorine upregulated the protein level of SHP-1, but did not affect the expression levels of other proteins (Fig. 5C). Lycorine failed to upregulate SHP-1 in HMEC cells (Supplementary Fig. S4). Moreover, Lycorine increased SHP-1 activity in the tested breast cancer cells (Fig. 5D). Furthermore, when SHP-1 was silenced with small-interference RNA (siRNA), the effects of Lycorine on the constitutive

Figure 4. Lycorine suppresses migration and invasion of breast cancer in vitro and in vivo

(A) MCF-7 cells were grown in a monolayer and then a wound was made by scraping with a pipette tip. After that cells were treated with 20 μM of Lycorine and images were taken at 0 h and 48 h. Scale bar = 50 μm. (B) MDA-MB-231 cells were grown in a monolayer and then a wound was made by scraping with a pipette tip. After that cells were treated with 20 μM of Lycorine and images were taken at 0 h and 24 h. Scale bar = 50 μm. (C) Relative migration areas of MCF-7 and MDA-MB-231 cells were analyzed by Image J software. (D–F) MCF-7 and MDA-MB-231 cells were treated with 20 μM of Lycorine, and the invasive capacities were determined by Transwell invasion assay. Representative photographs of invasive cells and quantification of invasive cells were shown. Scale bar = 100 μm. (G) Luciferase-expressing MDA-MB-231 cells were injected into the nude mice through the tail vein. Animals were randomized to receive either Lycorine (10 mg/kg/d) or DMSO at 1 week after injection. The Luciferase intensities were detected at the second, forth, and sixth week, respectively. Representative images of mice from bioluminescent imaging at the sixth week were shown and the luciferase intensities were detected. (F) Representative hematoxylin and eosin staining were shown and the number of metastatic foci in the lungs of each group was counted. Scale bar = 200 μm. Data are expressed as the mean ± SD of three independent experiments. *P < 0.05.
phosphorylation and DNA-binding activity of STAT3 was partly abolished (Fig. 5E,F). In addition, SHP-1 reduction inhibited IL-6-induced STAT3 activation in HMEC cells (Supplementary Fig. S5).

**Discussion**

Despite recent advances in diagnosis and treatment of breast cancer, its mortality rate still remains high. Conventional anti-cancer chemotherapies always associate with occurrence of side effects induced by the non-specific targeting of both normal and cancer cells [23]. Therefore, development of novel agents for the prevention and treatment of human breast cancer is urgently needed. Lycorine is a natural alkaloid that exists in the flowers and bulbs of Amaryllidaceae species, such as daffodils (Narcissus), snowdrops (Galanthus), and spider lilies (Lycoris) [24]. Lycorine has been shown to possess a potential anti-cancer effect and low toxicity in various malignant tumors [16–22]. Therefore, the role of Lycorine in breast cancer needs to be further clarified. In the present study, we found that Lycorine-induced apoptosis and suppressed proliferation, colony formation, migration, and invasion of breast cancer cells. Administration of Lycorine decreased both tumor weight and volume as well as lung metastasis. Mechanistically, Lycorine suppresses constitutive STAT3 phosphorylation through enhancing SHP-1 expression, and decreases the expressions of apoptosis- and invasion-related proteins. All these results demonstrate the unique anti-tumor abilities of Lycorine as a potential candidate for the treatment of breast cancer.

STAT3 activation has been associated with the survival, proliferation, metastasis, and chemoresistance of cancer cells [25–29]. STAT3 inhibitors have potential to be used in the treatment and prevention of cancer [30]. Previous study has reported that Lycorine targets STAT3 signal pathway to inhibit the growth and metastasis of hormone-refractory prostate cancer [22]. Therefore, whether the effects of Lycorine on the growth and metastasis of breast cancer are through inhibiting STAT3 signal pathway was explored. Our results showed that Lycorine significantly reduced the expression of p-STAT3 and DNA-binding activity in breast cancer cells. Lycorine also reduced the expressions of the STAT3-regulated proteins, such as anti-apoptotic proteins (Mcl-1 and Bcl-xL) and the invasion-related proteins (MMP-2 and MMP-9).

SHP-1, one of non-transmembrane phosphotyrosine phosphatases, plays a critical role in regulating cytokine/ protein tyrosine kinase-mediated signaling [31,32]. Extensive studies have...
demonstrated that the expression of SHP-1 is decreased in many malignant tumors and SHP-1 functions as a tumor suppressor [33]. The downregulated expression of SHP-1 was also found in estrogen-receptor negative breast cancer cell lines [34]. Ectopic expression of SHP-1 inhibits the growth of breast cancer cells partly by negative regulation of activated JAK kinase [35]. In addition, SHP-1 is required for receptor-mediated cytotoxic signaling that causes intracellular acidification and apoptosis in breast cancer cells [36]. There are many known substrates of SHP-1 in different types of cells, such as JAK2 and STAT5 in erythropoietic cells [37], c-KIT in hematopoietic cells [38] and TrkA in neuron cells [39]. However, except p-STAT3, few SHP-1 substrates have been report in breast cancer cells [40–43]. Our available data showed that Lycorine might target p-STAT3 partly by enhancing SHP-1 activity, but further studies are certainly necessary to explore the effects of Lycorine on other potential SHP-1 substrates in breast cancer cells. Although Lycorine showed little impact on the expressions of p-JAK2, SHP-2 and PTPIB, the role of other upstream kinases of STAT3 cannot be completely ruled out and further studies are needed.

Lycorine are thought to inhibit the proliferation of cancer cells through inducing cell cycle arrest and apoptosis. Li et al. [16] reported that Lycorine induces cell cycle arrest and apoptosis by activating caspase pathway in HL-60 cells. Cai et al. [20] showed that Lycorine also induces apoptosis through downregulating the expressions of Mcl-1 and Bcl-2 in human leukemia cells. Based on our study, Lycorine inhibited proliferation and induced apoptosis in breast cancer cells in vitro, but showed no apparent effect on cell cycle. Furthermore, Lycorine exerted little impact on HMECs, which are human normal mammary epithelial cells. Results from in vivo experiments also showed apparent anti-tumor activity of Lycorine toward breast cancer, with no marked changes in the body, liver, and spleen weights. These results suggest that the anti-tumor activity of Lycorine in breast cancer at physiologically achievable concentrations is moderate. In addition, Lycorine upregulated the expressions of critical factors in the caspase pathway, such as cleaved-caspase-3, cleaved-caspase-9, and cleaved-PARP. More importantly, a pan-caspase inhibitor (Z-VAD-FMK) reversed the effects of Lycorine on apoptosis and the expression of cleaved-caspase-3 in breast cancer cells. Lycorine also downregulated the expressions of the anti-apoptotic proteins Mcl-1 and Bcl-xL. These results show that Lycorine induces apoptosis in breast cancer cells, partially in a caspase-dependent manner.

MMPs are the main family of proteolytic enzymes that facilitate tumor cell migration by degrading the basement membrane and other components of the extracellular matrix (ECM) [44,45]. In advanced breast cancer, proteolytic enzymes such as MMP-2 and MMP-9 are overexpressed, which play important roles in ECM and BM degradation [46]. A recent study demonstrated that Lycorine inhibits the metastasis of prostate cancer partly through downregulating the expressions of MMP-2 and MMP-9 [22]. Consistent with this study, we found that Lycorine decreased the expressions of MMP-9 and MMP-2. These results indicate that the anti-migration and anti-invasion effects of Lycorine on breast cancer can be attributed to the inhibition of MMP-9 and MMP-2.

In conclusion, we demonstrate that Lycorine significantly inhibits the growth and metastasis of breast cancer by suppressing the constitutive STAT3 activation and downregulating the expressions of STAT3-regulated gene products. Although the role of Lycorine in breast cancer still needs to be further demonstrated in clinical trials, we believe that Lycorine may be a potential agent for the prevention and therapy of breast cancer.

### Supplementary Data

Supplementary data is available at Acta Biochimica et Biophysica Sinica online.

### References
