# Protective role of autophagy in matrine-induced gastric cancer cell death

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Received October 21, 2012; Accepted December 7, 2012

DOI: 10.3892/ijo.2013.1817

**Abstract.** Matrine has potent antitumor activity against a broad variety of cancer cells and our previous study showed that both autophagy and apoptosis were activated during matrine-induced gastric cancer cell death. The aim of the present study was to determine the significance of autophagy in antineoplastic effects of matrine and the molecular mechanism by which matrine induces autophagy in gastric cancer cells. Western blot analysis showed that exposure of gastric cancer cells to matrine resulted in the extent of autophagy increasing in a dose- and time-dependent manner by detecting microtubule-associated protein 1 light chain 3 (LC3). This induction was due to activation of autophagic flux, as supported using the lysosome inhibitor, bafilomycin A1, which produced an accumulation of LC3-II. Propidium iodide staining demonstrated that matrine induced cell death in a dose-dependent manner and the autophagy inhibitor 3-methyladenine (3-MA) or bafilomycin A1 enhanced lethality of matrine against gastric cancer cells. Moreover, after pretreatment with 3-MA, some of the gastric cancer cells treated with matrine exhibited prototypical characteristics of apoptosis by transmission electron microscopy. The ability of 3-MA to increase matrine-induced apoptosis was further confirmed by Annexin V-FITC/PI staining. Also, the combination of matrine and 3-MA was more potent than matrine alone in inhibiting the proliferation of SGC-7901 cells assessed by sulphorhodamine B assay. Furthermore, administration of the pan-caspase inhibitor zVAD-fmk or autophagy inducer rapamycin decreased the matrine-induced cell death. In addition, matrine treatment did not inhibit the phosphorylation of Akt and its downstream effectors mammalian target of rapamycin (mTOR) as well as p70 ribosomal protein S6 kinase (p70S6K), although the levels of the total Akt and mTOR were decreased. These results suggest that autophagy was activated as a protective mechanism against matrine-induced apoptosis and inhibition of autophagy may be an attractive strategy for enhancing the antitumor potential of matrine in gastric cancer.

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*Key words:* matrine, autophagy, apoptosis, gastric cancer, light chain 3, cell death

## Introduction

Gastric cancer is one of the most common types of cancer with approximately 989,600 new cases and 738,000 deaths worldwide in 2008 (1). Its mortality remains high as most patients are diagnosed at an advanced stage when the tumor is irresectable or metastatic. Although chemotherapy has made progress in the remission of this disease, drug resistance in the course of treatment has become more common. Therefore, new cytotoxic agents, especially natural compounds, or novel therapeutic strategies still need to be explored.

Matrine, one of the main alkaloid components extracted from a traditional Chinese herb, Sophora flavescens Ait, with a molecular formula of $C_{15}H_{24}N_2O$ (Fig. 1A), has been shown to possess various biological properties including anti-inflammatory (2-4), antiviral (5,6), antifibrotic (7,8), analgesic (9) and antiarrhythmic (10,11). Recent evidence indicates that matrine plays an important role in the treatment of tumors, without obvious toxicity or side-effects. Matrine also attenuates cancer cachexia-related symptoms in colon-26 tumor-bearing mice (12), and enhances patient immune functions and thus improves the quality of life (13). Our previous studies showed that matrine exhibited potent anticancer effects in gastric cancer and hepatocellular carcinoma cells (14,15). The mechanisms underlying the antineoplastic activity of matrine may stem from cell cycle arrest, inhibition of cell proliferation and induction of apoptosis, perhaps through the modulation of apoptosis- and/or proliferation-related genes and proteins, such as proliferating cell nuclear antigen, c-myc, apoptosis protein-activating factor, Bcl-2 family members, caspases, and Fas/FasL (13,16-21), indicating matrine-induced cell apoptosis can be triggered by the death receptor extrinsic pathway and/or the mitochondrial intrinsic pathway. Also, our previous research demonstrated that autophagy is involved in the antitumor effects of matrine on SGC-7901 human gastric cancer cells (14).

Autophagy is an intracellular lysosome-dependent catabolic process that is essential for maintaining cellular homeostasis through the turnover and elimination of defective or redundant proteins and damaged or aged organelles (22). Evidence suggests that autophagy may be important in the regulation of cancer development and progression, which is a double-edged sword in oncology (23-27). In addition, a large body of findings indicates that various anticancer therapies induce autophagy in different cancer cells (28,29). However, whether autophagy in response to therapies results in cell death or instead protects cancer cells from death is controversial. Under physiological conditions, autophagy is constitutively at low basal levels in every cell. A variety of environmental stresses, such as nutrient starvation, or anticancer drug treatment, can trigger dramatic enhancement of the level of autophagy acting as a cytoprotective response, resulting in adaptation and survival; however, autophagy may become a cell death mechanism if the amplitude of autophagy increases above a threshold level (30,31). In several cases, it is agreed that autophagic cell death, also defined as type II programmed cell death, is an important cell death process distinct from apoptosis (type I programmed cell death) (32,33). In our previous study, we found that both autophagy and apoptosis were activated during the matrine-induced death of SGC-7901 cells. Since the role of autophagy in anticancer treatment may depend on the nature of the cancer, the drug, or both (34), it is necessary to elucidate whether matrine-induced autophagy itself is responsible for cell killing, or protects cancer cells against matrine treatment by blocking the apoptosis. In addition, the exact mechanism by which matrine induces autophagy remains unclear. There are a number of signaling pathways involved in the regulation of autophagy. The class I phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase (p70S6K) signaling pathway has been studied extensively (35,36), and several anticancer compounds have been reported to induce autophagy through inhibiting the pathway (37-41). Therefore, in order to make matrine therapy for gastric cancer more efficacious and less toxic, we further clarified whether the signaling pathway is involved in the induction of autophagy triggered by matrine.

## Materials and methods

*Reagents and antibodies*. Reagents used included fetal bovine serum (Hyclone), dimethyl sulfoxide (DMSO) (Sigma, D2650), 3-methyladenine (3-MA) (Sigma, M9281), bafilomycin A1 (Sigma, B1793), rapamycin (Sigma, R8781), Z-Val-Ala-Asp fluoromethylketone (zVAD-fmk) (Sigma, C2105), matrine (Tianyuan Biologics Plant, Xi'an, China), Annexin V-FITC apoptosis detection kit (Invitrogen, V13242), PhosSTOP Phosphatase Inhibitor Cocktail (Roche, 04906845001), and SuperSignal West Pico Chemiluminescent Substrate (Pierce, NCI5080). Antibodies were obtained from the following sources: microtubule-associated protein 1 light chain 3 (LC3) (Abcam, ab51520), Akt (Cell Signaling Technology, 9272), phospho-Akt (Ser473) (Cell Signaling Technology, 4060), mTOR (Cell Signaling Technology, 2972), phospho-mTOR (Ser2448) (Cell Signaling Technology, 2971), p70S6K (Cell Signaling Technology, 9202), phospho-p70S6K (Thr389) (Cell Signaling Technology, 9205), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hangzhou Goodhere Biotech Co., AB-P-R 001), horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Zhongshan Goldenbridge Biotech Co., ZB-2301).

*Cell culture and treatments*. The human gastric cancer cell line SGC-7901 was obtained from the Cell Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 $\mu$g/ml of streptomycin at 37˚C in a 5% $CO_2$ incubator. The cells in mid-log phase were used in the experiments. Matrine (purity >99%) was dissolved in sterile double distilled water at a stock concentration of 40 mg/ml, and then diluted in RPMI-1640 medium to obtain the desired concentration. zVAD-fmk and bafilomycin A1 were dissolved in DMSO and control cells were similarly treated with DMSO to a maximum final concentration of 0.2%. This concentration of DMSO did not cause any adverse morphologic response. zVAD-fmk, rapamycin and bafilomycin A1 were added 1 h before matrine treatment. 3-MA was dissolved in heated sterile double distilled water to make a 100-mM stock solution and then added to the medium for a final concentration of 5 mM. After 3 h, matrine was added for treatment.

*Western blot analysis*. SGC-7901 cells were seeded into tissue culture flasks, allowed to attach 24 h and then exposed to matrine with or without specific inhibitors 3-MA, zVAD-fmk, rapamycin or bafilomycin A1. After the end of specified treatment periods, cells were lysed on ice with RIPA buffer for 20 min in the presence of PhosSTOP and then centrifuged at 12,000 rpm for 15 min at 4˚C. The supernatant was collected and stored in aliquots at -80˚C until analysis by western blotting. Fifty micrograms protein were loaded into each well and separated by electrophoresis through 8, 10 or 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (Millipore) using Trans-Blot Semi-Dry Cell or Mini Trans-Blot Cell apparatus (Bio-Rad). After blocking with 5% non-fat dry milk or bovine serum albumin in 1X TBST (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20) for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4˚C followed by incubation with HRP-conjugated goat anti-rabbit secondary antibodies for 1 h at room temperature. The specific protein bands were developed using SuperSignal West Pico Chemiluminescent Substrate and imaged using a VersaDoc imaging system (Bio-Rad).

*Propidium iodide staining assay*. Cells were trypsinized with 0.25% trypsin, collected and resuspended in 100 $\mu$l of precooled phosphate-buffered saline (PBS). After adding 1 $\mu$l of propidium iodide (PI) staining solution (100 $\mu$g/ml), cells were incubated for 20 min in the dark at room temperature, supplemented with 400 $\mu$l of binding buffer and analyzed using a flow cytometer (Beckman Coulter, USA).

*Transmission electron microscopy*. After 24 h of treatment, SGC-7901 cells were harvested by trypsinization, washed twice with PBS and fixed in 2.5% glutaraldehyde for 90 min at

room temperature, and post-fixed in 1% osmium tetraoxide for 30 min. After washing with PBS, the cells were progressively dehydrated in ascending grades of ethanol solutions (50, 70, 95 and 100%), and embedded in Epon 812 resin. The blocks were cut into ultra-thin sections with a microtome, and were then stained with saturated uranyl acetate and lead citrate. The ultrastructure of the cells was then examined in a transmission electron microscope (JEM-1230, Jeol, Japan).

*Annexin V-FITC/PI staining assay*. Following drug treatment, floating cells were collected and combined with adherent cells that were detached from culture dishes by treating with trypsin. Total cells were then washed with cold PBS twice and resuspended in 1X Annexin V binding buffer at a concentration of $1x10^6$ cells/ml. A single-cell suspension (100 $\mu$l) was stained with 5 $\mu$l Annexin V-FITC and 1 $\mu$l of PI for 15 min at room temperature in the dark. After the incubation period, 400 $\mu$l of 1X Annexin V binding buffer were added to each tube, followed by cytometric analysis (EPICS XL, Beckman Coulter). The extent of apoptosis was quantified as a percentage of Annexin V-positive cells, and all experiments were performed in triplicate.

*Sulphorhodamine B colorimetric assay*. To determine cell viability, the sulphorhodamine B (SRB) colorimetric assay, which estimates cell number indirectly by staining total cellular protein with the dye SRB, was used (42). SGC-7901 cells were seeded in 96-well flat bottom microtiter plates at a density of $5x10^3$ cells per well. Following treatment, the cells were fixed with 10% (w/v) trichloroacetic acid at 4˚C for 1 h, and then stained at room temperature for 20 min with 0.4% (w/v) SRB solution. The cells were subsequently washed with 1% acetic acid five times and dissolved in 150 $\mu$l of 10 mmol/l Tris base solution (pH 10.5). The absorbance value per well at 510 nM was read using an automatic multiwell spectrophotometer (PowerWave x, Bio-Tek Instruments Inc., USA). All SRB assays were repeated three times. Cell viability was calculated according to the formula: Cell viability (%) = A510 (sample)/A510 (control) x 100.

*Statistical analysis*. All data are expressed as the means ± standard deviation (SD). Statistical analysis was performed using the SPSS 16.0, and $p<0.05$ was considered to indicate statistically significant differences. The data were analyzed by ANOVA followed by Bonferroni t-test for multiple comparisons.

## Results

*Matrine induces LC3-II accumulation*. We have reported that matrine induces autophagy in SGC-7901 cells which relies on the observation of cell ultrastructural changes by transmission electron microscopy and the visualization of monodansylcadaverine-labeled autophagic vacuoles by fluorescence microscopy. Here, we further examined the expression levels of LC3 which exists in cells in two forms, LC3-I and LC3-II. LC3-I residing in the cytoplasm is conjugated to phosphatidylethanolamine to form LC3-II, which is closely associated with autophagosome membranes and serves as a reliable marker to monitor autophagy. Western blot analysis of proteins from matrine-treated cells revealed the presence of two bands (Fig. 1B). Weak bands corresponding to LC3-I were found in the untreated cells whereas LC3-II was undetectable. When the cells were treated with matrine at the concentration of 1.0 mg/ml, the expression levels of LC3-II significantly enhanced in a time-dependent manner. Furthermore, LC3-II was observed as early as 3 h posttreatment with matrine. Exposure of SGC-7901 cells to matrine also resulted in a concentration-dependent increase of LC3-II protein levels at each of the treatment points indicated (Fig. 1C).

*Effects of 3-MA and bafilomycin A1 on autophagy during matrine treatment*. The increase in LC3-II expression could reflect either increased formation of autophagosomes due to increases in autophagic activity, or reduced turnover of autophagosomes (43). To address the issue, we assessed the influence of matrine on LC3-II levels in the presence of bafilomycin A1 or 3-MA. Bafilomycin A1 is an inhibitor of the vacuolar-type ATPase that alters the pH of acidic compartments, which blocks the fusion of autophagosomes with lysosomes, thus preventing the autophagic degradation including that of LC3-II (44). 3-MA can inhibit autophagy due to suppression of class III phosphatidylinositol 3-kinase which is essential for the initiation of the early stages of autophagy (45). The addition of 3-MA was shown to decrease LC3-II levels in matrine-treated cells, while bafilomycin A1 had their LC3-II further accumulated during matrine treatment (Fig. 1D and E). The opposite effects of 3-MA and bafilomycin A1 on LC3 are due to the two inhibitors blocking autophagy at different stages; 3-MA inhibited autophagosome formation from the beginning, whereas bafilomycin A1 prevented degradation of LC3 in autophagolysosomes and in turn increased LC3 levels (46). These findings demonstrated that the LC3-II accumulation induced by matrine was a consequence of increased autophagosome formation, indicating that matrine increased autophagic flux, rather than prevented autophagic degradation, in SGC-7901 cells.

*Matrine induces cell death in a dose-dependent manner*. Cell death in response to matrine was quantified by PI staining and flow cytometry, and PI positive cells were counted as dead cells. SGC-7901 cells were treated with matrine at concentrations ranging from 0 to 2.0 mg/ml for 24 h. As shown in Fig. 2A, matrine killed gastric cancer cells in a dose-dependent manner. At the concentration of 2.0 mg/ml matrine, the cell death rate reached 35.67±4.82%.

*Inhibition of autophagy enhances matrine-induced cytotoxicity*. The above results showed that exposure of gastric cancer cells to matrine resulted in the extent of autophagy increasing in a dose- and time-dependent manner, which is positively correlated with the cytotoxic effect of matrine; hence, we further investigated whether matrine-induced cell death was mediated by autophagy. Furthermore, our previous study demonstrated that both apoptosis and autophagy were activated during matrine treatment (14), therefore it is necessary to further explore the interconnection between matrine-induced autophagy and apoptosis. To address these questions, we first determined whether inhibition of autophagy by 3-MA affects the cytotoxicity of matrine. The result showed that the cell death by flow cytometry in the gastric cells treated with matrine plus 3-MA sharply

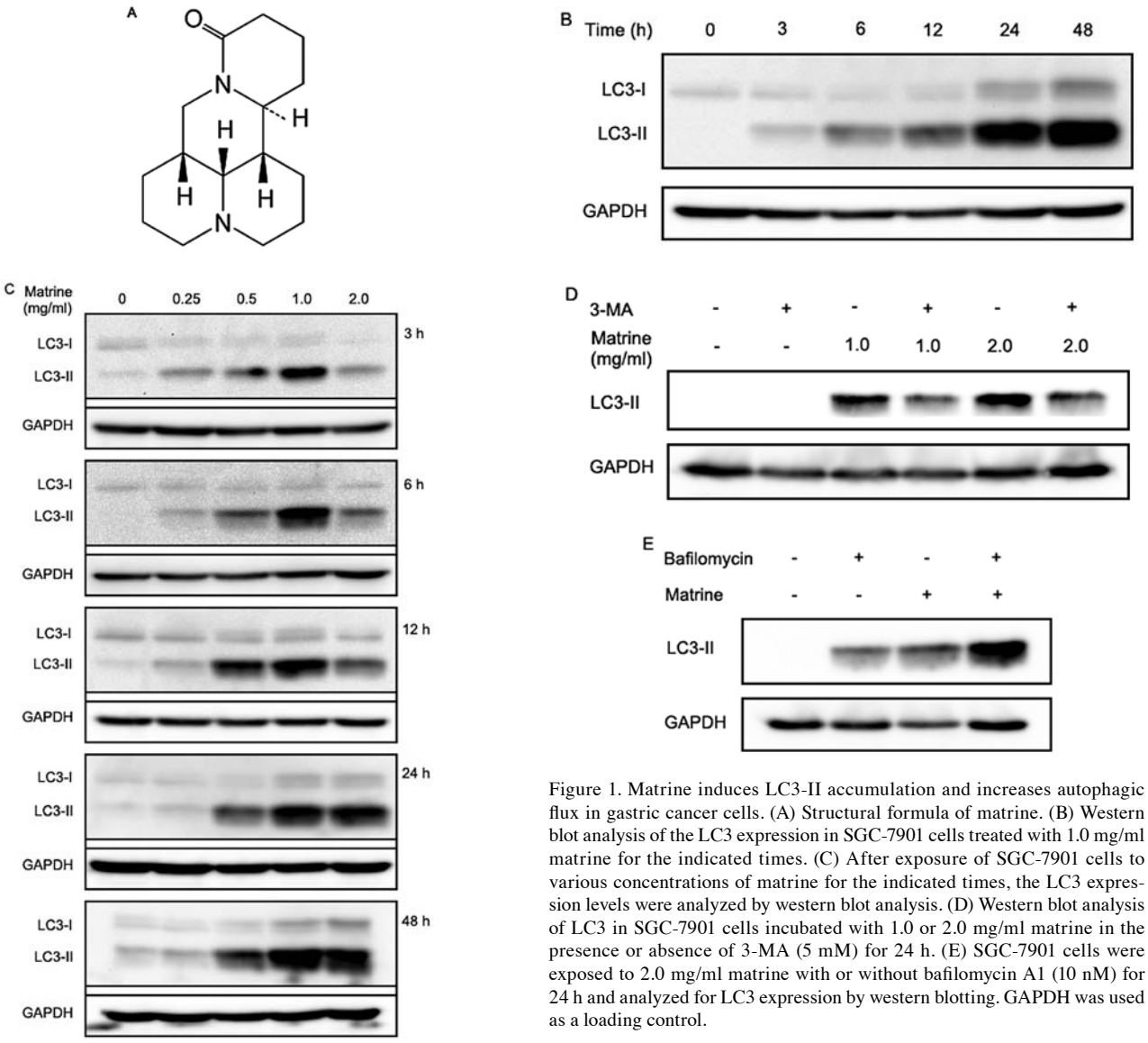


Figure 1. Matrine induces LC3-II accumulation and increases autophagic flux in gastric cancer cells. (A) Structural formula of matrine. (B) Western blot analysis of the LC3 expression in SGC-7901 cells treated with 1.0 mg/ml matrine for the indicated times. (C) After exposure of SGC-7901 cells to various concentrations of matrine for the indicated times, the LC3 expression levels were analyzed by western blot analysis. (D) Western blot analysis of LC3 in SGC-7901 cells incubated with 1.0 or 2.0 mg/ml matrine in the presence or absence of 3-MA (5 mM) for 24 h. (E) SGC-7901 cells were exposed to 2.0 mg/ml matrine with or without bafilomycin A1 (10 nM) for 24 h and analyzed for LC3 expression by western blotting. GAPDH was used as a loading control.

increased compared with matrine alone (Fig. 2B). At the same time, we used another inhibitor of autophagy at a late stage, bafilomycin A1, to further establish the role of autophagy in cell death induced by matrine. As shown in Fig. 2C, although bafilomycin A1 treatment alone had little effect on SGC-7901 cells, this agent similarly enhanced the cytotoxicity of matrine. Also, the combination of matrine with 3-MA acted cooperatively to decrease the viability of SGC-7901 cells when compared with matrine alone, measured by SRB assay (Fig. 2D). Markedly, when 3-MA inhibited matrine-induced autophagy, inspection of the cells co-treated with matrine and 3-MA using transmission electron microscopy revealed prototypical characteristics of apoptosis. As shown in Fig. 3, control cells without matrine exposure exhibited normal ultrastructural morphology. The cells treated with 1 mg/ml matrine alone showed presence of abundant autophagic vacuoles sequestrating cytoplasm and organelles and absence of chromatin changes. By contrast, after incubation with matrine in the presence of 3-MA for 24 h, the number of autophagic vacuoles decreased in the cells and some of these cells underwent apoptosis, which is characterized by cell shrinkage, plasma membrane blebbing, and chromatin condensation with margination of chromatin to the nuclear membrane. Similar effects were further confirmed by Annexin V-FITC/PI double staining that addition of 3-MA augmented matrine-induced apoptosis of gastric cancer cells compared to matrine treatment alone (Fig. 3E), indicating that matrine-induced autophagy may constitute a pro-survival compensatory mechanism, the inhibition of which drives more cells to die of apoptosis. Consistent with these observations, other studies also reported that matrine induced apoptosis in gastric cancer SGC-7901 cells (21) and MKN45 cells (20) via activation of crucial caspases such as caspase-3. Collectively, the results suggested that the cytotoxic effect of matrine on SGC-7901 cells is caused by apoptosis but not by autophagy which is a protective response by preventing cells from undergoing apoptosis, and autophagy inhibition could accelerate cell death induced by matrine.

*Blockade of apoptosis reduces matrine-induced cell death*. To further explore if matrine-induced cell death is apoptotic, the cells were pretreated with a pan-caspase inhibitor zVAD-fmk for 1 h followed by exposure to matrine for 24 h. zVAD-fmk is a widely used inhibitor in characterizing apoptotic cell death

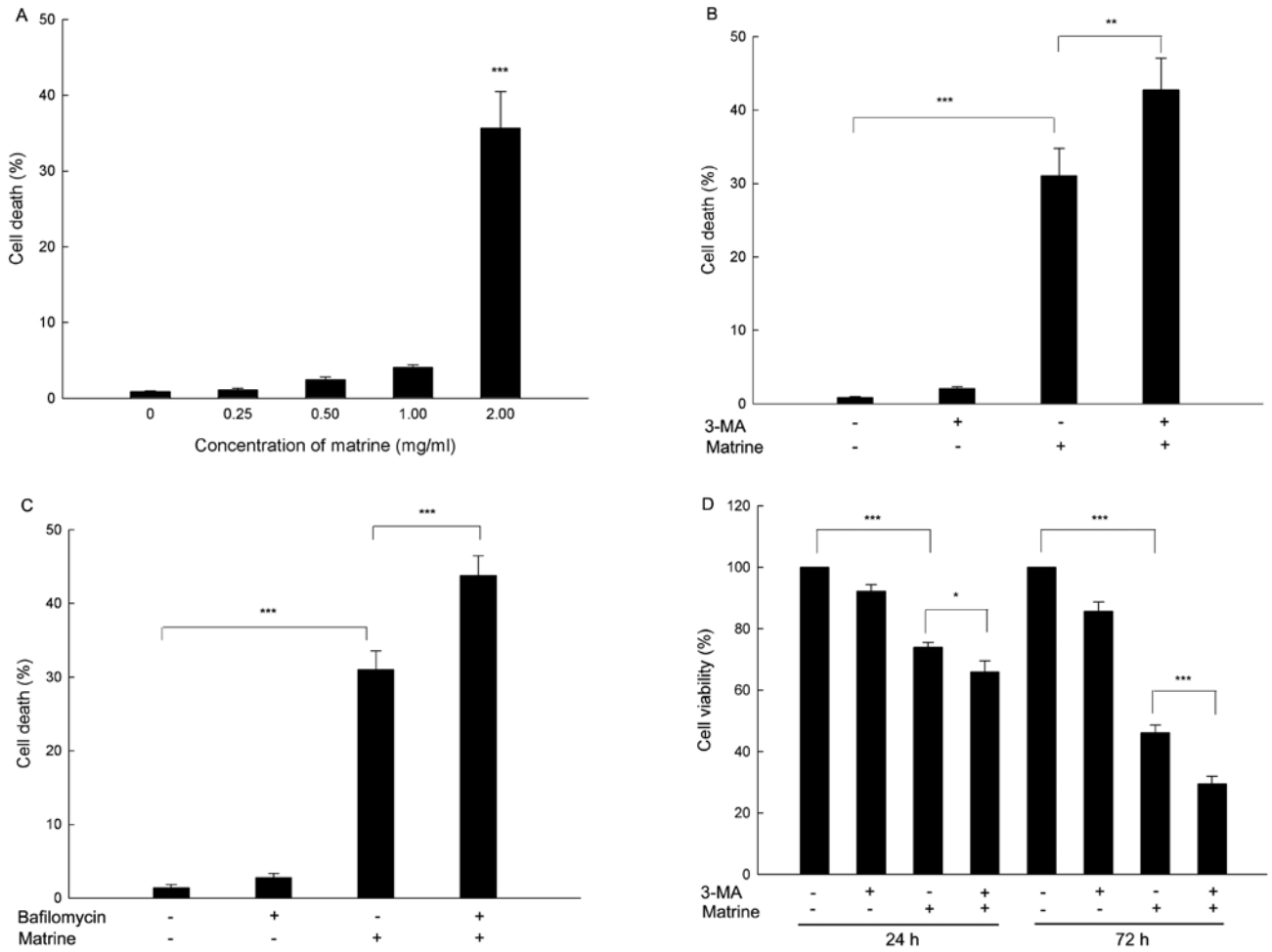


Figure 2. Matrine induces cell death and inhibition of autophagy increases cytotoxicity of matrine towards gastric cancer cells. (A) SGC-7901 cell death in response to matrine at various concentrations for 24 h was quantified by PI staining and flow cytometry. (B and C) Effects of 3-MA and bafilomycin A1 on the matrine-induced cell death. SGC-7901 cells were incubated for 24 h with matrine (2.0 mg/ml) in the presence or absence of autophagy inhibitor 3-MA (5 mM) or bafilomycin A1 (10 nM); cell death was determined by PI staining and flow cytometry. (D) Cell viability was assessed by sulphorhodamine B assay in SGC-7901 cells treated with 1 mg/ml matrine in the absence or presence of 3-MA (5 mM) for 24 and 72 h. Results are expressed as the means ± SD (n=3). $^{*}$p<0.05, $^{**}$p<0.01, $^{***}$p<0.001.

as it binds irreversibly to the active site of activated caspases and inhibits caspase-mediated apoptosis (47). As shown in Fig. 4A, matrine-induced cell death was markedly reversed by zVAD-fmk, which further demonstrated apoptosis was the major form of cell death induced by matrine and may be caspase-dependent.

*Enhancement of autophagy decreases matrine-induced cell death*. It has been suggested that excessive autophagy ultimately induces a type of cell death called autophagic cell death (32,48-50). Therefore, we hypothesized that if the cell death induced by matrine is indeed mediated by autophagy, the cytotoxicity of matrine is significantly enhanced after co-treatment with matrine and rapamycin which is known to be a potent autophagy inducer. As shown in Fig. 4B, however, rapamycin did not cause an additional increase of cell death quantified by flow cytometry, but instead attenuated the cell death.

*Matrine decreases total Akt level but increases Akt phosphorylation in gastric cancer cells*. The PI3K/Akt/mTOR/p70S6K signaling pathways are well-known pathways involved in the regulation of autophagy, and some anticancer agents are reported to block the pathways (37-41). Therefore, we examined the effect of matrine on the pathways, using western blotting. Akt is a downstream target of PI3K, which is phosphorylated at Tyr308 by phosphoinositide-dependent kinase 1 and at Ser473 by the mTOR complex 2 (mTORC2), resulting in its full activation (51). As shown in Fig. 5A, matrine-treated cells exhibited a dramatic increase in Akt phosphorylation at Ser473 in a dose-dependent manner. However, total Akt levels decreased in a dose-dependent manner after treatment with matrine at 3, 6 and 24 h, respectively, especially in the cells exposed to 2.0 mg/ml of matrine for 24 h. This was unexpected, as Akt activation is well known to suppress autophagy in mammalian cells. On the other hand, the increased Akt phosphorylation might, in part, limit clinical anticancer efficacy of matrine due to its suppression of apoptosis.

It has been reported that mTOR inhibitors such as rapamycin induce increased levels of phospho-Akt via negative feedback regulation of insulin receptor substrate-1 (52). As expected, rapamycin increased levels of phospho-Akt; notably, co-treatment with matrine and rapamycin was able to augment this counterproductive increase (Fig. 5B). The activation of Akt may be responsible for the finding that addition of rapamycin contributed to the decrease of the cell death induced by matrine (Fig. 4B).

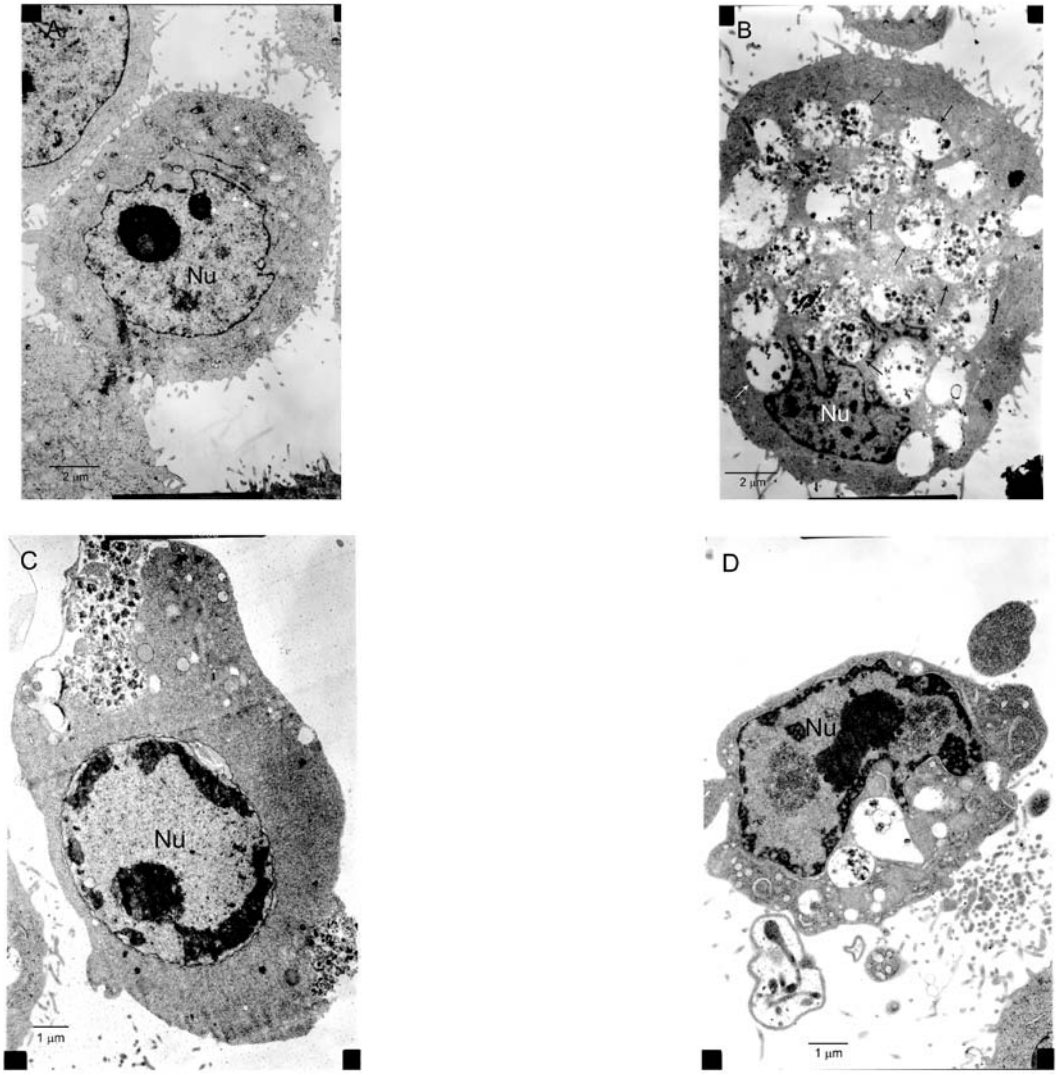


Figure 3. Blockade of autophagy drives more gastric cancer cells into apoptosis. (A-D) Ultrastructural observations in SGC-7901 cells by transmission electron microscope. (A) Untreated SGC-7901 cells exhibited normal morphology. (B) SGC-7901 cells treated with 1 mg/ml matrine for 24 h showed abundant autophagic vacuoles in cytoplasm. (C and D) Following pretreatment with 3-MA (5 mM), some of the matrine-treated cells showed characteristics of apoptosis. (A and B) magnification x4,000; (C) magnification x5,000; (D) magnification x6,000. Arrows, autophagic vacuoles; nu, nucleus. (E) Annexin V-FITC/PI double staining was performed to measure apoptotic ratios in SGC-7901 cells treated with 1 mg/ml matrine in the absence or presence of 5 mM 3-MA for 24 h. Results are expressed as the means ± SD (n=3). $^{*}p<0.05$, $^{***}p<0.001$.

*Matrine induces a slight increase in phosphorylation of mTOR and p70S6K in gastric cancer cells*. The serine/threonine kinase mTOR is a major downstream substrate of Akt and is at the core of two distinct multiprotein kinase complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 activation by the PI3K/Akt pathway results in phosphorylation and activation of p70S6K at Thr389 and mTORC2 has a role in activating Akt through Ser473 phosphorylation (53). As shown in Fig. 6, matrine slightly increased phosphorylation of mTOR at Ser2448 in gastric cancer cells at 6 h, which was accompanied by a slight decrease in total mTOR protein in a dose-dependent manner. We also observed matrine treatment for 6 h modestly increased p70S6K at Thr389 phosphorylation and without any effect on the total p70S6K level. These

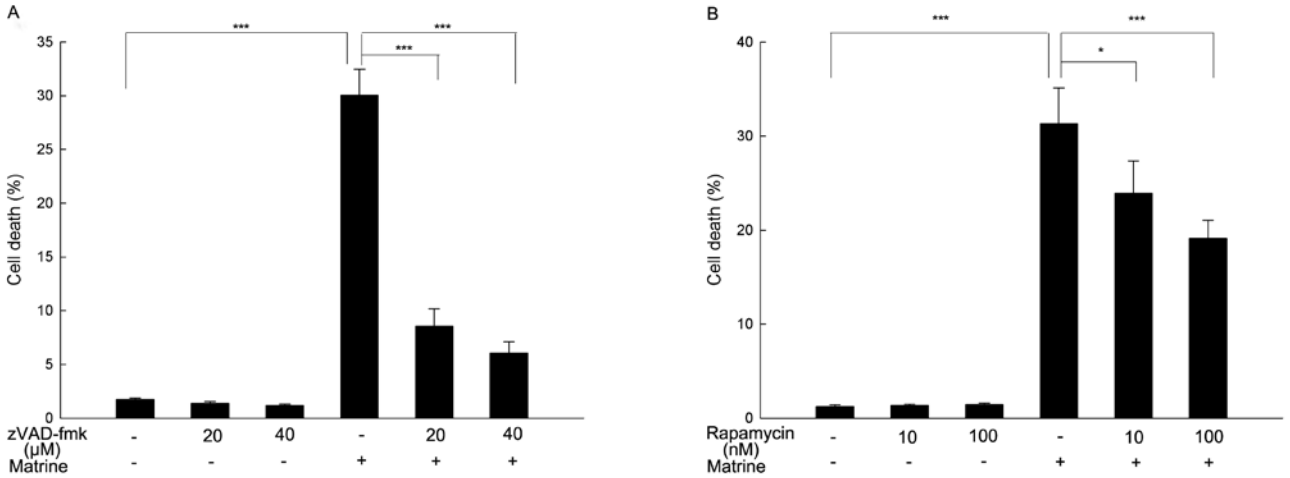


Figure 4. Inhibition of apoptosis or enhancement of autophagy decreases matrine-induced cell death. SGC-7901 cells were incubated for 24 h with 2.0 mg/ml matrine in the presence or absence of the pan-caspase inhibitor zVAD-fmk (20 or 40 $\mu$M) or autophagy inducer rapamycin (10 or 100 nM), and the cell death was assessed by PI staining and flow cytometry. Results are expressed as the means ± SD (n=3). $^{*}$p<0.05, $^{***}$p<0.001.

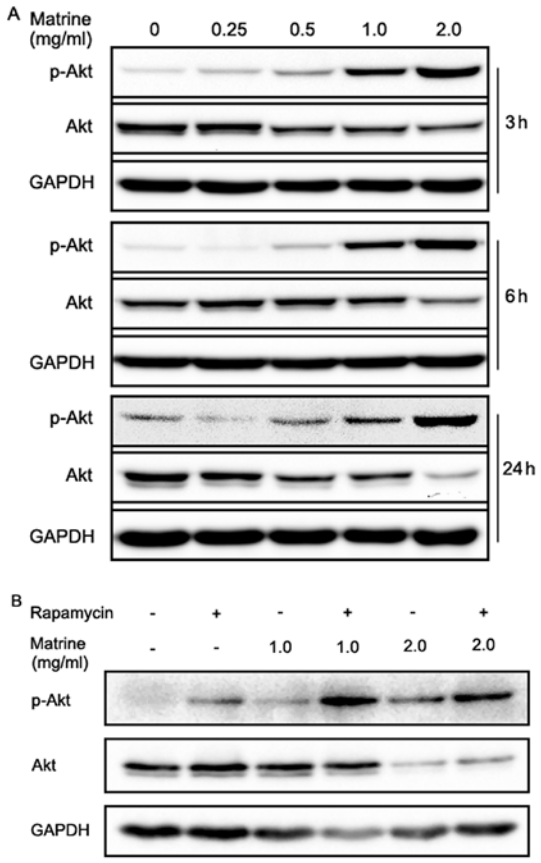


Figure 5. Matrine decreases total Akt level but increases Akt phosphorylation in gastric cancer cells. (A) Western blot analysis of phospho-Akt (Ser473) and total Akt from lysates of SGC-7901 cells treated with increasing concentrations of matrine for 3, 6 and 24 h, respectively. (B) Western blot analysis of phospho-Akt (Ser473) and total Akt from lysates of SGC-7901 cells treated with 1.0 or 2.0 mg/ml of matrine for 24 h in the presence or absence of rapamycin (10 nM). GAPDH was used as a loading control.

results were counterproductive, as activated mTOR is generally considered to be involved in the negative control of mammalian autophagy.

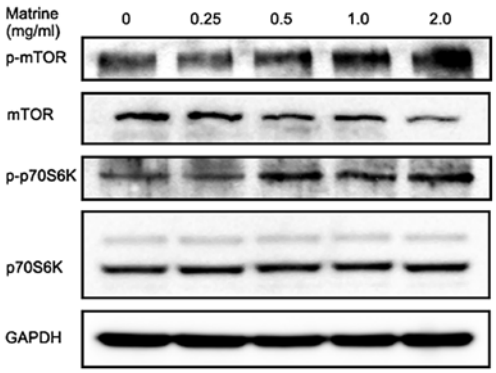


Figure 6. Matrine slightly increases phosphorylation of mTOR and p70S6K in gastric cancer cells. Western blot analysis of phospho-mTOR (Ser2448), total mTOR, phospho-p70S6K (Thr389) and total p70S6K from lysates of SGC-7901 cells treated with various concentrations of matrine for 6 h. GAPDH was used as a loading control.

## Discussion

*Sophora flavescens Ait* is listed in Chinese Pharmacopoeia and has been widely used in China for medicinal purposes. Our previous study showed that matrine purified from *Sophora flavescens Ait* has potent antitumor activity against gastric cancer cells, and is a strong inducer of autophagy as well as of apoptosis (14). In the present study, we further demonstrated that the degree of autophagy in gastric cancer cells by detecting LC3 increased in a dose- and time-dependent manner after matrine treatment. From these results, we considered two possibilities: matrine-induced autophagy may be a mechanism of the antitumor effect of matrine, resulting in autophagic cell death, or a stress adaptation leading to survival of tumor cells during matrine treatment. Here, we employed several pharmacological inhibitors of the autophagic or apoptotic process to determine the significance of autophagy in antineoplastic properties of matrine. The data demonstrated that the presence of autophagic inhibitors such as 3-MA or bafilomycin A1 enhances lethality of matrine against gastric cancer cells. Notably, after pretreatment

with 3-MA, some of the cells exposed to matrine displayed classic apoptotic morphology. Accordingly, 3-MA increased matrine-induced apoptosis evidenced by flow cytometry using Annexin V-FITC/PI staining, suggesting that matrine-induced autophagy protects cells from apoptosis and could be a target for enhancing its antitumor effects. Moreover, co-treatment with matrine plus 3-MA significantly enhanced the antiproliferative effect on SGC-7901 cells in comparison with matrine alone. In addition, administration of the pan-caspase inhibitor zVAD-fmk decreased the cell death rate in matrine-treated cells, indicating that apoptosis is the major form of cell death induced by matrine, consistent with other studies that matrine induces apoptosis in gallbladder carcinoma (13), retinoblastoma (54), multiple myeloma (55), osteosarcoma (56), pancreatic cancer (16), hepatocellular carcinoma (57), and gastric cancer cells (20,21). Autophagy appears to function as a prosurvival mechanism perhaps through the degradation and recycling of the damaged cellular proteins and organelles caused by matrine treatment, which protects cancer cells from matrine-mediated apoptosis and causes resistance to matrine therapy. Therefore, inhibition of autophagy potentiates the apoptosis-inducing and anticancer activity of matrine, suggesting a strategy in the clinic to augment the antineoplastic efficacy of matrine by combining matrine with an autophagic inhibitor.

Increasing evidence indicates that various anticancer therapies induce autophagy in different cancer cells. Furthermore, autophagic cell death can be activated in cancer cell lines in response to various agents used in cancer treatment, such as Rhabdastrellic acid-A (58), arsenic trioxide (59), silibinin (60), zoledronic acid (61), berberine (37), α-mangostin (62), dasatinib (38), resveratrol (63), fisetin (64), 5-fluorouracil (65) and triptolide (39). However, despite these examples, we did not find that matrine-treated cell death is executed by autophagy; we found that cell death is accompanied by features of autophagy which acts primordially as a cytoprotective mechanism during matrine treatment, consistent with the notion that autophagy, in most cases, constitutes an attempt of dying cells to cope with lethal stress rather than a mechanism to execute cell demise (66). A recent study clearly showed that autophagic cell death may be unlikely to exist as a phenomenon since a large collection of clinically used or experimental anticancer agents (~1400 compounds) did not contain a single compound that would trigger cell death through induction of autophagy (67). In addition, there is a growing body of literature supporting the idea that autophagy is activated in tumor cells as a prosurvival mechanism against cytotoxic agents and may therefore favor chemoresistance. For instance, compound C (40), resveratrol (68), quercetin (41) timosaponin A-III (69), and celecoxib (70) induce protective autophagy in various cancer cells, and acquired cisplatin resistance in human lung adenocarcinoma cells is associated with enhanced autophagy (71). Therefore, the term 'autophagic cell death' is considered to be a misnomer in that it describes a reality in which cells die with autophagy but not by autophagy (66,72). Since the term 'autophagic cell death' is highly prone to misinterpretation, the Nomenclature Committee of Cell Death in 2012 suggested that the term should only be used in the rare occasions where definite proof can be provided that cell death is mediated by autophagy (73).

Although there is extensive knowledge on the mechanisms of induction of apoptosis by matrine (13,16,19-21), very little is known about the specific mechanisms of matrine-induced autophagy. Diverse signaling pathways have been reported in the regulation of autophagy in mammalian cells in response to multiple forms of cellular stress including starvation, hypoxia, radiation or chemical insults (74). Of these, the Akt/mTOR pathway is considered a typical negative regulator for the initiation of autophagy (41). Accumulating anticancer agents have been documented to trigger the cellular autophagic process by suppressing the pathway, such as berberine (37), dasatinib (38), triptolide (39), compound C (40) and quercetin (41). Therefore, we sought to determine the potential involvement of the Akt/mTOR pathway in the matrine-induced autophagic process. However, the results showed that matrine treatment did not inhibit the phosphorylation of Akt (Ser473) and its downstream effectors mTOR (Ser2448) as well as p70S6K (Thr389), although the levels of the total Akt and mTOR were decreased. Accordingly, it appeared that the Akt/mTOR pathway was not involved in the induction of autophagy in the matrine-treated SGC-7901 cells. Therefore, another main pathway might be involved in the autophagy induction process. For example, autophagy can also be activated by the Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-related kinase (ERK) pathway (30,75). It has been reported that autophagy induced by triptolide or curcumin is associated with the activation of the pathway (39,76). Also, arsenic-induced autophagy appears to require activation of the MEK/ERK pathway but not the Akt/mTOR pathways (77). Hence, further investigations are required to gain insights into the potential involvement of other signaling pathways and the precise underlying mechanism of matrine-induced autophagy. In addition, matrine treatment increased the phosphorylation of Akt at Ser473 in a dose-dependent manner, which may contribute to the resistance of gastric cancer cells to matrine, thus attenuating their potential antitumor activity. In line with the unexpected result, treatment with arsenic trioxide or sorafenib also enhanced the levels of the phosphorylation of Akt in several types of cancer cells (77-79). Despite inducing Akt phosphorylation, matrine significantly suppressed the proliferation of gastric cancer cells in our previous and present study (14). It is possible that combinations of matrine with Akt inhibitor may provide a potential approach to augment the antitumor properties of matrine.

In conclusion, the present study suggests that matrine-induced autophagy in gastric cancer cells is an adaptive response that delays the eventual cell death, and blockade of autophagy could be a promising strategy to improve the ability of matrine to kill gastric cancer cells. In addition, the Akt/mTOR/p70S6K signaling pathway might not be involved in the induction of autophagy in the matrine-treated SGC-7901 cells.

**Acknowledgements**

This study was supported by the National Natural Science Foundation of China (grant no. 30870364) and the Science and Technology Support Program of Gansu province, China (grant no. 0708NKCA129).

## References


1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Zhang B, Liu ZY, Li YY, *et al*: Antiinflammatory effects of matrine in LPS-induced acute lung injury in mice. Eur J Pharm Sci 44: 573-579, 2011.
3. Liu JY, Hu JH, Zhu QG, Li FQ, Wang J and Sun HJ: Effect of matrine on the expression of substance P receptor and inflammatory cytokines production in human skin keratinocytes and fibroblasts. Int Immunopharmacol 7: 816-823, 2007.
4. Suo Z, Liu Y, Ferreri M, *et al*: Impact of matrine on inflammation related factors in rat intestinal microvascular endothelial cells. J Ethnopharmacol 125: 404-409, 2009.
5. Li CQ, Zhu YT, Zhang FX, *et al*: Anti-HBV effect of liposome-encapsulated matrine in vitro and in vivo. World J Gastroenterol 11: 426-428, 2005.
6. Long Y, Lin XT, Zeng KL and Zhang L: Efficacy of intramuscular matrine in the treatment of chronic hepatitis B. Hepatobiliary Pancreat Dis Int 3: 69-72, 2004.
7. Zhang JP, Zhang M, Zhou JP, *et al*: Antifibrotic effects of matrine on in vitro and in vivo models of liver fibrosis in rats. Acta Pharmacol Sin 22: 183-186, 2001.
8. Zhang JP, Zhang M, Jin C, *et al*: Matrine inhibits production and actions of fibrogenic cytokines released by mouse peritoneal macrophages. Acta Pharmacol Sin 22: 765-768, 2001.
9. Yin LL and Zhu XZ: The involvement of central cholinergic system in (+)-matrine-induced antinociception in mice. Pharmacol Biochem Behav 80: 419-425, 2005.
10. Li X, Chu W, Liu J, *et al*: Antiarrhythmic properties of long-term treatment with matrine in arrhythmic rat induced by coronary ligation. Biol Pharm Bull 32: 1521-1526, 2009.
11. Zhou Y, Xu W, Han R, *et al*: Matrine inhibits pacing induced atrial fibrillation by modulating I(KM3) and I(Ca-L). Int J Biol Sci 8: 150-158, 2012.
12. Zhang Y, Wang S, Li Y, Xiao Z, Hu Z and Zhang J: Sophocarpine and matrine inhibit the production of TNF-alpha and IL-6 in murine macrophages and prevent cachexia-related symptoms induced by colon26 adenocarcinoma in mice. Int Immunopharmacol 8: 1767-1772, 2008.
13. Zhang Z, Wang X, Wu W, *et al*: Effects of matrine on proliferation and apoptosis in gallbladder carcinoma cells (GBC-SD). Phytother Res 26: 932-937, 2012.
14. Zhang J, Li Y, Chen X, *et al*: Autophagy is involved in anticancer effects of matrine on SGC-7901 human gastric cancer cells. Oncol Rep 26: 115-124, 2011.
15. Zhang JQ, Li YM, Liu T, *et al*: Antitumor effect of matrine in human hepatoma G2 cells by inducing apoptosis and autophagy. World J Gastroenterol 16: 4281-4290, 2010.
16. Liu T, Song Y, Chen H, Pan S and Sun X: Matrine inhibits proliferation and induces apoptosis of pancreatic cancer cells in vitro and in vivo. Biol Pharm Bull 33: 1740-1745, 2010.
17. Qin XG, Hua Z, Shuang W, Wang YH and Cui YD: Effects of matrine on HepG2 cell proliferation and expression of tumor relevant proteins in vitro. Pharm Biol 48: 275-281, 2010.
18. Zhang LP, Jiang JK, Tam JW, *et al*: Effects of Matrine on proliferation and differentiation in K-562 cells. Leuk Res 25: 793-800, 2001.
19. Jiang H, Hou C, Zhang S, *et al*: Matrine upregulates the cell cycle protein E2F-1 and triggers apoptosis via the mitochondrial pathway in K562 cells. Eur J Pharmacol 559: 98-108, 2007.
20. Luo C, Zhu Y, Jiang T, *et al*: Matrine induced gastric cancer MKN45 cells apoptosis via increasing pro-apoptotic molecules of Bcl-2 family. Toxicology 229: 245-252, 2007.
21. Dai ZJ, Gao J, Ji ZZ, *et al*: Matrine induces apoptosis in gastric carcinoma cells via alteration of Fas/FasL and activation of caspase-3. J Ethnopharmacol 123: 91-96, 2009.
22. Li Y, Zhang J, Chen X, *et al*: Molecular machinery of autophagy and its implication in cancer. Am J Med Sci 343: 155-161, 2012.
23. Apel A, Zentgraf H, Buchler MW and Herr I: Autophagy-A double-edged sword in oncology. Int J Cancer 125: 991-995, 2009.
24. Hippert MM, O'Toole PS and Thorburn A: Autophagy in cancer: good, bad, or both? Cancer Res 66: 9349-9351, 2006.
25. Mathew R, Kongara S, Beaudoin B, *et al*: Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev 21: 1367-1381, 2007.
26. Degenhardt K, Mathew R, Beaudoin B, *et al*: Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10: 51-64, 2006.
27. Mathew R, Karantza-Wadsworth V and White E: Role of autophagy in cancer. Nat Rev Cancer 7: 961-967, 2007.
28. Kondo Y and Kondo S: Autophagy and cancer therapy. Autophagy 2: 85-90, 2006.
29. Eisenberg-Lerner A and Kimchi A: The paradox of autophagy and its implication in cancer etiology and therapy. Apoptosis 14: 376-391, 2009.
30. Yang Z and Klionsky DJ: Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 22: 124-131, 2010.
31. Levine B and Kroemer G: Autophagy in the pathogenesis of disease. Cell 132: 27-42, 2008.
32. Gozuacik D and Kimchi A: Autophagy and cell death. Curr Top Dev Biol 78: 217-245, 2007.
33. Sun Y and Peng ZL: Programmed cell death and cancer. Postgrad Med J 85: 134-140, 2009.
34. Marx J: Autophagy: is it cancer's friend or foe? Science 312: 1160-1161, 2006.
35. Yang Z and Klionsky DJ: An overview of the molecular mechanism of autophagy. Curr Top Microbiol Immunol 335: 1-32, 2009.
36. Glick D, Barth S and Macleod KF: Autophagy: cellular and molecular mechanisms. J Pathol 221: 3-12, 2010.
37. Wang N, Feng Y, Zhu M, *et al*: Berberine induces autophagic cell death and mitochondrial apoptosis in liver cancer cells: the cellular mechanism. J Cell Biochem 111: 1426-1436, 2010.
38. Le XF, Mao W, Lu Z, Carter BZ and Bast RC Jr: Dasatinib induces autophagic cell death in human ovarian cancer. Cancer 116: 4980-4990, 2010.
39. Mujumdar N, Mackenzie TN, Dudeja V, *et al*: Triptolide induces cell death in pancreatic cancer cells by apoptotic and autophagic pathways. Gastroenterology 139: 598-608, 2010.
40. Vucicevic L, Misirkic M, Janjetovic K, *et al*: Compound C induces protective autophagy in cancer cells through AMPK inhibition-independent blockade of Akt/mTOR pathway. Autophagy 7: 40-50, 2011.
41. Wang K, Liu R, Li J, *et al*: Quercetin induces protective autophagy in gastric cancer cells: involvement of Akt-mTOR- and hypoxia-induced factor 1alpha-mediated signaling. Autophagy 7: 966-978, 2011.
42. Skehan P, Storeng R, Scudiero D, *et al*: New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107-1112, 1990.
43. Klionsky DJ, Abeliovich H, Agostinis P, *et al*: Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4: 151-175, 2008.
44. Klionsky DJ, Baehrecke EH, Brumell JH, *et al*: A comprehensive glossary of autophagy-related molecules and processes (2nd edition). Autophagy 7: 1273-1294, 2011.
45. Wu YT, Tan HL, Shui G, *et al*: Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. J Biol Chem 285: 10850-10861, 2010.
46. Periyasamy-Thandavan S, Jiang M, Wei Q, Smith R, Yin XM and Dong Z: Autophagy is cytoprotective during cisplatin injury of renal proximal tubular cells. Kidney Int 74: 631-640, 2008.
47. Chen SY, Chiu LY, Maa MC, Wang JS, Chien CL and Lin WW: zVAD-induced autophagic cell death requires c-Src-dependent ERK and JNK activation and reactive oxygen species generation. Autophagy 7: 217-228, 2011.
48. Hotchkiss RS, Strasser A, McDunn JE and Swanson PE: Cell death. N Engl J Med 361: 1570-1583, 2009.
49. Scarlatti F, Granata R, Meijer AJ and Codogno P: Does autophagy have a license to kill mammalian cells? Cell Death Differ 16: 12-20, 2009.
50. Levine B and Yuan J: Autophagy in cell death: an innocent convict? J Clin Invest 115: 2679-2688, 2005.
51. Coughlin CM, Johnston DS, Strahs A, *et al*: Approaches and limitations of phosphatidylinositol-3-kinase pathway activation status as a predictive biomarker in the clinical development of targeted therapy. Breast Cancer Res Treat 124: 1-11, 2010.
52. O'Reilly KE, Rojo F, She QB, *et al*: mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 66: 1500-1508, 2006.
53. Guertin DA and Sabatini DM: Defining the role of mTOR in cancer. Cancer Cell 12: 9-22, 2007.
54. Zhao B, Li B, Bai S, *et al*: Effects of matrine on proliferation and apoptosis of cultured retinoblastoma cells. Graefes Arch Clin Exp Ophthalmol 250: 897-905, 2012.

55. Han Y, Zhang S, Wu J, *et al*: Matrine induces apoptosis of human multiple myeloma cells via activation of the mitochondrial pathway. Leuk Lymphoma 51: 1337-1346, 2010.
56. Liang CZ, Zhang JK, Shi Z, Liu B, Shen CQ and Tao HM: Matrine induces caspase-dependent apoptosis in human osteosarcoma cells in vitro and in vivo through the upregulation of Bax and Fas/FasL and downregulation of Bcl-2. Cancer Chemother Pharmacol 69: 317-331, 2012.
57. Ma L, Wen S, Zhan Y, He Y, Liu X and Jiang J: Anticancer effects of the Chinese medicine matrine on murine hepatocellular carcinoma cells. Planta Med 74: 245-251, 2008.
58. Li DD, Guo JF, Huang JJ, *et al*: Rhabdastrellic acid-A induced autophagy-associated cell death through blocking Akt pathway in human cancer cells. PLoS One 5: e12176, 2010.
59. Kanzawa T, Zhang L, Xiao L, Germano IM, Kondo Y and Kondo S: Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3. Oncogene 24: 980-991, 2005.
60. Duan WJ, Li QS, Xia MY, Tashiro S, Onodera S and Ikejima T: Silibinin activated p53 and induced autophagic death in human fibrosarcoma HT1080 cells via reactive oxygen species-p38 and c-Jun N-terminal kinase pathways. Biol Pharm Bull 34: 47-53, 2011.
61. Lin JF, Lin YC, Lin YH, *et al*: Zoledronic acid induces autophagic cell death in human prostate cancer cells. J Urol 185: 1490-1496, 2011.
62. Chao AC, Hsu YL, Liu CK and Kuo PL: α-Mangostin, a dietary xanthone, induces autophagic cell death by activating the AMP-activated protein kinase pathway in glioblastoma cells. J Agric Food Chem 59: 2086-2096, 2011.
63. Puissant A, Robert G, Fenouille N, *et al*: Resveratrol promotes autophagic cell death in chronic myelogenous leukemia cells via JNK-mediated p62/SQSTM1 expression and AMPK activation. Cancer Res 70: 1042-1052, 2010.
64. Suh Y, Afaq F, Khan N, Johnson JJ, Khusro FH and Mukhtar H: Fisetin induces autophagic cell death through suppression of mTOR signaling pathway in prostate cancer cells. Carcinogenesis 31: 1424-1433, 2010.
65. Xiong HY, Guo XL, Bu XX, *et al*: Autophagic cell death induced by 5-FU in Bax or PUMA deficient human colon cancer cell. Cancer Lett 288: 68-74, 2010.
66. Shen S, Kepp O and Kroemer G: The end of autophagic cell death? Autophagy 8: 1-3, 2012.
67. Shen S, Kepp O, Michaud M, *et al*: Association and dissociation of autophagy, apoptosis and necrosis by systematic chemical study. Oncogene 30: 4544-4556, 2011.
68. Li J, Qin Z and Liang Z: The prosurvival role of autophagy in resveratrol-induced cytotoxicity in human U251 glioma cells. BMC Cancer 9: 215, 2009.
69. Sy LK, Yan SC, Lok CN, Man RY and Che CM: Timosaponin A-III induces autophagy preceding mitochondria-mediated apoptosis in HeLa cancer cells. Cancer Res 68: 10229-10237, 2008.
70. Huang S and Sinicrope FA: Celecoxib-induced apoptosis is enhanced by ABT-737 and by inhibition of autophagy in human colorectal cancer cells. Autophagy 6: 256-269, 2010.
71. Ren JH, He WS, Nong L, *et al*: Acquired cisplatin resistance in human lung adenocarcinoma cells is associated with enhanced autophagy. Cancer Biother Radiopharm 25: 75-80, 2010.
72. Kroemer G and Levine B: Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol 9: 1004-1010, 2008.
73. Galluzzi L, Vitale I, Abrams JM, *et al*: Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 19: 107-120, 2012.
74. Kroemer G, Marino G and Levine B: Autophagy and the integrated stress response. Mol Cell 40: 280-293, 2010.
75. Pattingre S, Bauvy C and Codogno P: Amino acids interfere with the ERK1/2-dependent control of macroautophagy by controlling the activation of Raf-1 in human colon cancer HT-29 cells. J Biol Chem 278: 16667-16674, 2003.
76. Aoki H, Takada Y, Kondo S, Sawaya R, Aggarwal BB and Kondo Y: Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: role of Akt and extracellular signal-regulated kinase signaling pathways. Mol Pharmacol 72: 29-39, 2007.
77. Goussetis DJ, Altman JK, Glaser H, McNeer JL, Tallman MS and Platanias LC: Autophagy is a critical mechanism for the induction of the antileukemic effects of arsenic trioxide. J Biol Chem 285: 29989-29997, 2010.
78. Qian W, Liu J, Jin J, Ni W and Xu W: Arsenic trioxide induces not only apoptosis but also autophagic cell death in leukemia cell lines via up-regulation of Beclin-1. Leuk Res 31: 329-339, 2007.
79. Shimizu S, Takehara T, Hikita H, *et al*: Inhibition of autophagy potentiates the antitumor effect of the multikinase inhibitor sorafenib in hepatocellular carcinoma. Int J Cancer 131: 548-557, 2012.