Narciclasine Induces Autophagy-Mediated Gastric Cancer Cell Apoptosis Through The Akt/mTOR Signaling Pathway

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Abstract

**Background:** Gastric cancer is a common gastrointestinal cancer and currently has the third highest mortality rate. According to research, the natural compound narciclasine has a variety of biological activities. The aim of the present study was to investigate the effect of narciclasine on gastric cancer cells and its molecular mechanisms, to determine whether this compound could be a novel therapy to treat gastric cancer.

**Methods:** MTT and clone assays were employed to detect the proliferation of gastric cancer cells. The apoptosis rate was detected by flow cytometry. The formation of autophagosomes and autophagosomal lysosomes was observed by transmission electron microscopy and laser confocal scanning microscopy. Western blotting was used to detect the expression of apoptosis, autophagy and Akt/mTOR pathway related proteins.

**Results:** In this study, we found that narciclasine could inhibit the proliferation of the gastric cancer cells, and promote gastric cancer cells apoptosis. Further experiments showed that narciclasine promoted the expression of autophagy proteins LC3-II, Atg-5, Beclin-1, reduced the expression of the autophagy transporter p62, and increased autophagic flux. By using the autophagy inhibitors 3-MA and CQ, it was shown that narciclasine can induces autophagy-mediated apoptosis in gastric cancer cells. Finally, we found that narciclasine had no significant effects on the total Akt and mTOR contents of gastric cancer cells, it involved autophagy in gastric cancer cells by reduce the phosphorylation levels of p-Akt and p-mTOR.

**Conclusions:** Taken together, narciclasine can induce autophagy-dependent apoptosis in gastric cancer cells by inhibiting the phosphorylation level of Akt/mTOR and thus reducing gastric cancer cell proliferation.

**Background**

Gastric cancer is a common cancer of the gastrointestinal tract. According to global cancer data in 2018, the new morbidity and mortality rates of people with gastric cancer were about 1.03 million and 780,000, respectively, ranking it the second most common among 36 types of cancer. Compared with 2012, both new cases and mortality were significantly increased[1–2]. Although surgical treatment, radiotherapy and chemotherapy of gastric cancer have made some progress in the past 10 years, the overall mortality rate has not been significantly reduced. The main reasons are the low surgical resection rate, the significant side effects of chemotherapy and the high recurrence rate after chemotherapy. Therefore, it is urgent to find a new way to treat gastric cancer.

Cell autophagy is a process in which cells use lysosomes to degrade their damaged organelles and macromolecular substances under the control of autophagy related genes (Atg), and is a self-protection mechanism of cells[3–4]. Under physiological conditions, autophagy can provide energy to the body and maintain intracellular homeostasis by degrading aging proteins and damaged organelles in cells[5].
Autophagy is also closely related with the occurrence and development of tumors. To some extent, autophagy can make tumor cells better adapt to external stress and contribute to the proliferation, invasion and drug resistance of tumor cells [6–7]. On the other hand, tumor cells can induce autophagic death when autophagy activation increases or continues to occur [8–9]. Therefore, the development of drugs that promote autophagy of gastric cancer cells is one potentially important mechanism to improve the survival rate of gastric cancer patients.

Narciclasine is a plant growth inhibitor isolated from the mucus secreted from narcissus bulbs. In 1967, Ceriotti et al. isolated narciclasine for the first time and in 1997 Rigby et al. completed the first organic synthesis of this compound [10–11]. Many studies in recent years have shown that narciclasine has various biological activities such as anti-inflammatory [12], inhibition of angiogenesis [13], anti-virus [14], anti-tumor effects [15], and so on. Therefore, the aim of the present study was to investigate the effect of narciclasine on gastric cancer cells and its molecular mechanisms. We selected moderately and poorly differentiated BGC-823 and SGC-7901 gastric cancer cells, highly differentiated MGC-803 and MKN28 cells, and human gastric mucosal epithelial GES-1 cells for investigation.

In the present study, we found that narciclasine could inhibit the proliferation of gastric cancer cells by stimulating autophagy, and its potential mechanism is to inhibit phosphorylation of the Akt/mTOR signaling pathway and the activation of autophagy, thus promoting apoptosis of gastric cancer cells.

**Materials And Methods**

**Reagents and cell culture**

Narciclasine was purchased from Chengdu Herbpurify CO., LTD (Chengdu, China), with a purity ≥ 99.9%. The human gastric cancer cell lines BGC-823, MGC-803, GES-1, MKN28 and SGC-7901 were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China): Roswell Park Memorial Institute 1640 (RPMI 1640), fetal bovine serum (FBS), penicillin and streptomycin Gibco Life Technologies (NY, US); chloroquine (CQ) and 3-methyladenine (3-MA) Selleck Chemicals (Texas, US); and insulin from Sigma-Aldrich (CA, US); AktiVIII was purchased from Macklin Inc (Beijing, China). Human gastric cancer cells were cultured in RPMI 1640 medium (containing 10% FBS and 2.5% penicillin-streptomycin), and cultured in a constant temperature incubator at 37°C and 5% CO₂ saturation humidity.

**MTT assay and colony formation assay**

Gastric cancer MGC-803, BGC-823, SGC-7901, MKN28 and GES-1 cells grown in the logarithmic phase were inoculated into 96-well plates with 5 × 10³ cells per well and 5 replicates in each group. The cells were treated with narciclasine. After 24 h, 10 µL of MTT (C0009, Beyotime Biotechnology, Shanghai, China) solution (5 mg/mL) was added to each well, which were placed in an incubator for 4 h, then aspirated to discard the MTT-containing culture solution. 150 µL of DMSO was added to each well, which
were shaken gently for 5–10 min on a mechanical shaker. The OD value of each well was read at 570 nm on a microplate reader.

Two hundred gastric cancer MGC-803 and BGC-823 cells were inoculated onto 12-well plates and culture medium containing final concentrations of 0.5 µM and 1 µM narciclasine were added, respectively. An equal amount of drug-free culture solution was added to the blank group and cells used after 14 days. Cells were fixed in 4% paraformaldehyde for 20 min, washed three times with PBS, and stained with 0.1% crystal violet (Sigma-Aldrich, CA, US) for 30 min, again washed three times with PBS, photographed and the number of clones in each group determined.

Flow cytometry assay

The gastric cancer cells were harvested after centrifugation after 24 h of treatment with narciclasine and resuspended in 500 µL of 1 × binding buffer. Five µL annexin V-FITC was added to the cell suspension first, then 10 µL PI (Annexin V-FITC Detection Kit, Beyotime Biotechnology, Shanghai, China) was added to the suspension. The cells were evenly mixed and then incubated in a 4°C refrigerator for 10 min in the dark. Cells were detected by flow cytometry (Accuri C6, Becton-Dickinson, US).

Transmission electron microscopy

After gastric cancer cells \((2 \times 10^5 - 1 \times 10^6)\) were treated with narciclasine for 24 h they were harvested, fixed with 2.5% glutaraldehyde in a volume > 20 times the sample volume for 4 h, rinsed with 0.1 MPBS, fixed with 1% osmic acid for 1 h, rinsed in double distilled H2O for 10–15 min twice and finally fixed/stained with 2% uranium acetate for 30 min. They then underwent gradient dehydration with alcohol, pure acetone + (1:1) penetrating embedding agent treatment, temperature gradient polymerization in an oven, ultra-thin sectioning using a microtome, uranium acetate-lead citrate staining, before finally being examined using a transmission electron microscope (Philips Co. Ltd., Netherlands).

Laser confocal scanning microscope observation

First, mRFP-GFP-tagged LC3 (tfLC3) (Hanbio Biotechnology Co. Ltd, Shanghai, China) gastric cancer cells were infected with adenovirus for 24 h (MOI = 300) and then fixed in formaldehyde at room temperature for 20 min. 5 µL of glycerol-PBS was added dropwise to each glass slide, which was then immersed in the cell culture medium and fixed. Any fluorescence changes of GFP and RFP in cells were observed using a laser confocal scanning microscope (Olympus Co., Ltd., Japan), and the number of autolysosomes was calculated after Merge.

Western blotting

Gastric cancer cells were lysed with RAPI lysate (Beyotime Biotechnology, Shanghai, China) and centrifuged at 4°C for 30 min at 14,000 rpm to concentrate and collect the proteins. The protein concentration in each sample was determined using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). The proteins were electrophoresed with 10% SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore, MA, US), blocked with 5% skimmed milk for 2 h,
and washed 3 times in TBST buffered saline. They were then incubated with Bax, Bcl-2, cleaved-PARP, LC3-II, Beclin1, p62, Akt, p-Akt, mTOR, p-mTOR (1:1000, Abcam, Cambridge, MA, USA) monoclonal antibodies overnight at 4ºC. A TBST buffered salt membrane was washed 3 times and IgG antibodies 1:2,000 dilution (MultiSciences, Shanghai, China) were incubated at room temperature for 1 h. TBST buffer salt was washed 3 times and an ECL chemiluminescence kit (Beyotime Biotechnology, Shanghai, China) used for color development in a dark room, then gel images were collected and analyzed. Quantity One software was used to analyze the corresponding gray value of each image strip.

**Statistical analysis**

Each experiment was independently repeated 3 times. All values were presented as mean ± standard deviation (SD). GraphPad Prism ver. 6.0 software (La Jolla, CA, US) was used for all statistical analyses. Potential differences were first analyzed using one-way ANOVA followed by the Newman-Keuls test. A *P*-value < 0.05 was considered to be a statistically significant difference.

**Results**

**Narciclasine inhibits proliferation and promotes apoptosis of gastric cancer cells**

In order to investigate the inhibitory effect of narciclasine (Fig. 1A) on the proliferation of gastric cancer cells, we used the MTT assay to detect the viability of gastric cancer and gastric mucosal cells, 24 h and 48 h after treatment with narciclasine at concentrations of 0.5 µM, 1 µM, 2 µM, 4 µM and 8 µM. The results showed that different concentrations of narciclasine significantly inhibited proliferation of gastric cancer cells BGC-823, SGC-7901, MGC-803 and MKN28, and also in a dose-dependent manner, but it had a weak toxic effect on gastric mucosal cells GES-1 (Fig. 1B-C). Based on the IC_{50} values of gastric cancer cells at 24 h (0.6406 µM for BGC-823, 0.6414 µM for SGC-7901, 0.4806 µM for MGC-803, 0.7013 µM for MKN28), we choose the gastric cancer cells BGC-823 and MGC-803 for the next experiments. Similarly, our cell cloning investigations showed that the number of cell clones in the narciclasine group was significantly less than in the control group (Fig. 1D-F). To further clarify whether the inhibition of narciclasine on proliferation of gastric cancer cells was related to apoptosis, flow cytometry was used to detect apoptosis as shown in Fig. 2A-C. The degree of apoptosis of gastric cancer cells in the treatment groups exposed to 0.5 µM and 1 µM narciclasine was significantly increased. In addition, compared with the control group, the Bcl-2 protein expression in the narciclasine treatment group was significantly decreased, while the expression of Bax and cleaved-PARP were increased. (Fig. 2D-F). These results showed that narciclasine could inhibit proliferation and promote the apoptosis of gastric cancer cells.

**Narciclasine promotes autophagy in gastric cancer cells**

Autophagy is an important pathway for anticancer effect[16], so we explored whether narciclasine could induce autophagy in gastric cancer cells. Firstly, we used western blotting to detect expression of the
autophagy proteins LC3-II, Atg-5, p62, and Beclin1 and the results are shown in Fig. 3A-C. The expression of the autophagy proteins LC3-II, Atg-5, and Beclin1 in the narcicasline treatment group was significantly increased and the expression of the autophagosome transporter p62 was decreased compared to control group. Similarly, we also observed using electron microscopy that the number of autophagosomes in the narcicasline treatment group was significantly higher than in the control group (Fig. 3D). Although the expression of LC-II, Atg-5 and Beclin1 increased as did autophagy after treatment with narcicasline, this does not fully indicate that narcicasline can enhance autophagy of gastric cancer cells, because when a the combination of autophagy and lysosome is inhibited, the above situation can also occur. Thus, we used mRFP-GFP-tagged LC3 to track the level of autophagosome-lysosome binding[17]. The results revealed that the number of autophagolysosomes in the gastric cancer cell mass of the narcicasline-treated group was significantly higher than that in the control group (Fig. 4A-C), which suggested that the autophagosomes could successfully bind to lysosomes instead of being blocked. To demonstrate further that narcicasline could induce an increase in the number of autophagosomes of gastric cancer cells and promote the binding of autophagosomes to lysosomes (i.e., promote autophagy), we used 3-MA (an autophagosome formation inhibitor) and CQ (autophagosome lysosomal binding inhibitor) and the results are shown in Fig. 4D-E. Narcicasline combined treatment with 3-MA markedly decreased the LC3-II/LC3-I ratio compared with narcicasline-treated gastric cancer cells, and combinatorial treatment with CQ resulted accumulation of LC3-II/LC3-I ratio, indicating that 3-MA and CQ can reverse part of the role of narcicasline in promoting autophagy. In addition, we also found that 3-MA and CQ could reverse the expression of p62 (Fig. 4F-G), which indicated that 3-MA and CQ could reduce the role of narcicasline in promoting the dissolution of p62 in lysosomes. Thus, narcicasline can enhance autophagy in gastric cancer cells.

**Narcicasline induces apoptosis by enhancing autophagy of gastric cancer cells**

Although autophagy and apoptosis are two forms of programmed cell death, they can co-exist or promote each other when driven by certain external stimuli[18]. Next, we explored whether the promotion of apoptosis by narcicasline was related to enhanced autophagy effects. First, we used the autophagy inhibitor 3-MA to detect apoptosis of gastric cancer cells after inhibiting autophagy and the results are presented in Fig. 5A-C, 3-MA could reduce apoptosis caused by narcicasline. Moreover, the 3-MA extremely rescued narcicasline mediated downregulation of Bax and cleaved-PARP, and promoted the expression of Bcl-2. (Fig. 5F-H). Similarly, the results of MTT experiments revealed that the gactric cancer cells activity more strengther when combined with 3-MA treatment (Fig. 5D-E), suggesting that autophagy inhibitors can reduce the pro-apoptosis and anti-proliferation effects of narcicasline on gastric cancer cells. This proves from the reverse point of view that narcicasline promotes gastric cancer apoptosis and inhibits proliferation by enhancing autophagy.

**Narcicasline promotes autophagy-induced apoptosis in gastric cancer cells through Akt/mTOR**
Many studies have shown that Akt/mTOR is closely related to autophagy[19–20], so we used western blotting to determine whether narciclasine had an effect on total Akt and mTOR, p-AKT and p-mTOR in gastric cancer cells. We found that narciclasine significantly inhibited levels of p-AKT and p-mTOR, but not Akt and mTOR total protein levels (Fig. 6A, D-E). To demonstrate further the role of Akt/mTOR dephosphorylation in narciclasine-induced autophagy, we treated gastric cancer cells with insulin (Akt activator) to reactivate Akt. The results showed that the insulin rescued narciclasine mediated downregulation of p-AKT and p-mTOR, and decreased the expression of LC3-II(Fig. 6B, F-G). Interestingly, when we used AKTiVIII (Akt/mTOR inhibitors) combined with narciclasine to treat gastric cancer cells, the expression level of LC3-II/C3-I was significantly increased compared with the narciclasine group, while the expression levels of p-AKT and p-mTOR decreased(Fig. 6C, H-I). In addition, our results revealed that the apoptosis rate of gastric cancer cells in the narciclasine combined with insulin group was less than in the narciclasine group, and that insulin could reduce the effects of narciclasine in inhibiting the proliferation of gastric cancer cells (Fig. 6J-N). In summary, the above results indicate that narciclasine activates gastric cancer cell autophagy by inhibiting the phosphorylation level of Akt/mTOR, thereby promoting gastric cancer cell apoptosis and inhibiting cell proliferation.

**Discussion**

It is well known that many natural compounds have strong anti-tumor effects, such as vincristine, irinotecan, etoposide and paclitaxel, etc., and they often have the characteristics of low toxicity, high efficiency, cost-effectiveness and wide sources[21–23]. Narciclasine is abundant present in fresh bulbs of natural plants such as Lycoris radiata, tiger ear, water ghost coke, snowflake genus, summer snowflake and yellow Lycoris radiata[14]. Studies have shown that narciclasine can act highly selectively on glioblastoma multiforme and inhibit the growth of Hela tumor cells[24–25]. The results of the present study found that narciclasine at a low concentration could significantly inhibit the proliferation in GC-823, MGC-803, MKN28 and SGC-7901 cells and inhibit colony formation of the gastric cancer BGC-823 and MGC-803 cells. According to research, narciclasine is highly selective for tumor cells, and its toxic effect on normal fibroblasts is 250 times lower than that of tumor cells; therefore it is unlikely to cause apoptosis of normal fibroblasts at therapeutic doses[26]. It is noteworthy that we found narciclasine had a weak toxic effect on gastric mucosal cells GES-1, but highly selective toxic effect on gastric cancer cells, indicating that narciclasine has great potential to be developed as an anti-gastric cancer drug.

Research has shown in human breast cancer MCF-7 cells and PC-3 prostate cells that narciclasine can activate Caspase-8 and Caspase-10 receptor pathways and induce apoptosis of tumor cells[27]. In the present study, we also found that narciclasine could promote the apoptosis of gastric cancer cells, inhibit the expression of Bcl-2 protein and increase the expression of Bax and cleaved-PARP protein, suggesting that narciclasine may activate the apoptosis pathway in gastric cancer cells. In addition, we confirmed that narciclasine can enhance the autophagy of gastric cancer cells, for example by the use of autophagy inhibitors.
Several relationships co-exist between autophagy and apoptosis: autophagy and apoptosis work together to promote cell death, with autophagy and apoptosis antagonizing each other; autophagy provides cells with the energy necessary for survival and inhibits their apoptosis; and autophagy promotes the transformation of cells to apoptosis[28–29]. Cao et al.[30] found that narciclasine could inhibit breast cancer cell proliferation by mediating autophagy-dependent apoptosis via AMPK-ULK1 signaling. In our study, we used 3-MA to inhibit autophagy and found that narciclasine induced a decrease in apoptosis of gastric cancer cells. Therefore, it is speculated that narciclasine promotes apoptosis of gastric cancer cells by activating autophagy. The interaction between Beclin1 and Bcl-2 is one of the common regulatory modes of autophagy and apoptosis[31]. Because there is a BH3 domain on Beclin1, Bcl-2 and Bcl-xl, other apoptosis factors can affect autophagy and apoptosis activity by binding to BH3[32]. When the expression level of Beclin1 increases, it can release the apoptosis promoting protein bak/Bax from its attachment to Bcl-2, thus promoting apoptosis. On the other hand, decreased Bcl-2 expression can also lead to more Beclin1-dependent autophagy[33–34]. Although this study confirmed that narciclasine can inhibit the expression of Bcl-2 and promote the expression of Beclin1, whether or not narciclasine mediated autophagic apoptosis of gastric cancer cells is related to the Beclin1-Bcl-2 regulatory mode needs further verification in future studies.

As early as 1975, Carrasco et al. found that narciclasine could effectively inhibit the biosynthesis of eukaryotic ribosome proteins and play an anti-tumor role in experiments on rabbit reticular cells and non-cell lines: the mechanism of action was to bind to the 60S ribosome group to inhibit the formation of chemical bonds[35]. In addition, narciclasine can also regulate the Rho / Rho kinase / LIM kinase conlin signaling pathway, strengthen the activity of GTPase RhoA and can induce the formation of actin stress fibers[24]. Akt is a serine/threonine protein kinase, also known as protein kinase B. When Akt is activated, it will regulate a large number of transcription factors and activate multiple substrates, including the target proteins of rapamycin (mTOR), thereby stimulating cell autophagy, proliferation and inhibition of apoptosis. When exploring the mechanism of narciclasine against gastric cancer, we found that narciclasine could inhibit the expression of p-Akt and p-mTOR. When we activated Akt with insulin, it could save the phosphorylation level of p-Akt and p-mTOR, and reduce the degree of autophagy and apoptosis of gastric cancer cells, suggesting that narciclasine-induced inhibition of the phosphorylation level of Akt/mTOR is one of its important mechanisms of action against gastric cancer. But the anti-tumor effect of drugs often involves multiple signaling pathways. In the future, we will clarify the anti-tumor effects of narciclasine acting through multiple pathways and multiple levels. In addition, the anti-tumor effect and mechanisms of action of narciclasine in vivo also need to be verified.

Conclusions

The results of our in vitro studies indicate that the natural compound narciclasine can mediate autophagy-dependent apoptosis of gastric cancer cells by inhibiting the phosphorylation level of Akt/mTOR. This research work will hopefully lead to the development of narciclasine into a much-needed novel targeted drug for the treatment of gastric cancer.
Abbreviations
NCS: Narciclasine; 3-MA: 3-Methyladenine; CQ: Chloroquine; LC3: Light chain 3; p62: Sequestosome 1/p62; Atg-5: Autophagy-related gene 5

Declarations

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Not Applicable.

Authors’ contributions
D.H.M. and Y.Y.F. wrote the main manuscript text, Y.Y.F., H.X., L.X., L.Y. and T.Y.L. prepared figures 1-4; D.H.M., Y.Y.F. and H.X. prepared figures 5-6, and T.Y.L. provide funding.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not Applicable. This study did not involve human participants and animals.

Consent for publication
Not Applicable.

Competing interests
The authors declare that they have no competing interests.

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