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*Original Article*

# Ginkgolic acids (C15 and C17) induce autophagy and apoptosis in gastric carcinoma HGC cells by inducing ER stress

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**Abstract:** Objective: This paper show that Ginkgolic acids can induce cell death in gastric cancer cell line HGC via a combination of autophagy and apoptosis pathway. Methods: The survival curves of the GAs (C15, C17) of the HGC were measured by MTT assay, and the expression of apoptosis associated proteins caspase-3, 8, 9 and the autophagy-associated protein 5, beclin 1 and microtubule-associated protein 1A/1B-light chain 3 in the GA-treated HGC cells were all measured by western blot analysis. The terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay was used to monitor the level of apoptosis in the GA-treated cells, and Transmission Electron Microscopy (TEM) was used to monitor the level of autophagy in the GA-treated cells. Results: MTT assays showed that both C15 and C17 inhibited HGC cell proliferation in a time-dependent and dose-dependent manner, and WB showed that the ER stress-, autophagy-, and apoptosis-related proteins were all increased. TUNEL, and TEM analysis show that the GAs induced both apoptosis and autophagy in the HGC cells. After CHOP expression was knocked down by small interfering RNA (siRNA), both C15 and C17 ginkgolic acid-treated cells showed increased ER stress-related protein expression, moreover, the LC3-II conversion and beclin-1 expression were reduced. After inhibition of ER stress by 4-PBA, both autophagy-related protein and apoptosis-related protein expression was decreased. After treatment with the autophagy inhibitor 3MA, both ER stress-related protein and apoptosis-related protein expression was decreased. After treatment with the c-Jun N-terminal kinase (JNK) pathway downregulator SP600125, the expression of beclin-1 and LC3-II conversion were reduced. Conclusions: The results suggest that both C15 and C17 ginkgolic acids increased ER stress upstream to regulate autophagy and apoptosis by three branch pathways (ATF6, inositol-requiring enzyme 1 (IRE1), and PKR-like ER protein kinase (PERK)). The IRE1-JNK pathway plays an important role in the crosstalk between ER stress and autophagy.

**Keywords:** Ginkgolic acids, endoplasmic reticulum stress, autophagy, apoptosis

## Introduction

Despite consistent progress in surgery and the integrated treatments of chemotherapy [1, 2], radiotherapy [3, 4], gene therapy [1] and immune therapy [5, 6], cancer is still the second leading cause of death, just following heart disease, and cancer is expected to be the leading cause of death in the next few years, with gastric cancer being the third most common cancer worldwide [7]. Currently, some studies have demonstrated that ginkgolic acids are a potential anticancer agent without a clear mechanism [8, 9].

Natural ginkgolic acids are a mixed extraction from the leaves, nuts and episperm of the Ginkgo biloba plant. According to the 6th position's side chain carbon atom number, naturally mixed ginkgolic acids can be derived into five forms, namely, 15:0, 15:1, 17:1, 17:2, and 13:0 ginkgolic acids [10]. It has been reported that

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mixed ginkgolic acids have anticancer activity against hepatoblastoma cells [11], but whether ginkgolic acid has anti-gastric cancer activity and which mixed ginkgolic acid extraction is most powerful is still unclear.

The endoplasmic reticulum (ER), which is associated with membrane protein modification, folding and assembly, is a conservative organelle in eukaryotic cells [12]. Sometimes, the protein is misfolded or unfolded in the endoplasmic reticulum when the cell is in a hard situation, such as hypoxia or $Ca^{2+}$ imbalance [13-15]. The unfolded protein response (UPR) is activated after the accumulation of misfolded and unfolded proteins reaches the threshold, and the three signaling pathways of ER stress (the PERK, ATF6, and IRE1 pathways) are differentially or selectively regulated by the sensors [16, 17]. The PERK pathway phosphorylates eIF2α [18], IRE1 splices XBP-1 [19], and ATF6 is proteolyzed to release its amino terminus (ATF6-N) [20]. All the regulation determines cell survival; in general, persistent, intense ER stress induces apoptosis or initiates autophagy [21]. Therefore, research on ER stress and autophagy in ginkgolic acid-induced apoptosis is worthwhile.

In this study, we focused on the changes in ER stress in gastric carcinoma HGC cells after treatment with C15 and C17 ginkgolic acids. Our findings show the basis for C15 and C17 ginkgolic acids as candidates for gastric carcinoma treatment.

## Methods

### *Materials*

The human gastric carcinoma cell line HGC-27 was obtained from The Cancer Cell Repository of Shanghai Cell Bank (Shanghai, China). Fetal bovine serum (FBS), trypsin, EDTA-2Na and DMEM were purchased from Gibco (NY, USA). All supplies for cell culture were obtained from Costar Corning (NY, USA).

Ginkgolic acids (GAs C15 and C17) were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). 3-Methyladenine (3-MA), SP600125, which is a specific inhibitor of c-Jun N-terminal kinase (JNK), and sodium phenylbutyrate (4-PBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The siRNA specific for CHOP was purchased from GenePharma (Shanghai, China). Lipofectamine 2000 was obtained from Invitrogen (CA, USA). The HRP-conjugated goat anti-beta-actin antibody and anti-p-eIF2α, anti-ATF6, anti-GRP78, anti-XBP1, and anti-CHOP were purchased from Santa Cruz (Texas, USA). Rabbit polyclonal anti-LC3, anti-PERK, anti-eIF2α, and anti-JNK antibodies were purchased from Boster (Wuhan, China). Rabbit monoclonal anti-p-JNK antibody was purchased from Cell Signaling Technology (Beverly, MA, USA), and anti-cleaved caspase-3, p-bcl-2 and p-PERK were purchased from ImmunoWay (Newark, DE, USA). HRP-conjugated goat anti-rabbit and HRP-conjugated goat-anti-mouse antibodies were purchased from CWBio (Shanghai, China). Alexa Fluor 488-conjugated goat anti-mouse antibody and Cy3-conjugated goat anti-mouse antibody were purchased from KPL (Washington, DC, USA). The Luminata and PVDF membranes were purchased from Millipore (MA, USA). 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) and DMSO were purchased from Amresco (PA, USA).

### *Cell culture*

HGC cells were cultured with 10% (v/v) FBS DMEM and with antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37°C with 5% $CO_2$ and 100% humidity. When the cells reached approximately 50-70% confluence, the cells were treated with GAs as follows.

### *GA administration*

When the HGC cells were in the exponential phase, which was usually approximately 50-70% confluent, the cells were collected and then cultured in 96-well, 24-well or 6-well plates at a certain concentration ($1\times10^4$/ml). The cells were cultured for 24 hours under certain conditions (10% FBS in DMEM, 37°C, and 5% $CO_2$). The following day, various concentrations of the two different GAs (0 μg/ml, 3.125 μg/ml, 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml). C15 and C17, were administered to HGC cells. The cells were cultured for the following days, and at each time point of Days 1, 2, 3, 4, and 5, the cells were harvested with the different protocols for a different analysis.

*MTT analysis*

At each scheduled harvest time point, each well of the plates was administered 20 μl MTT (5 mg/ml, PBS) and then cultured for another 4 hours. Then, 150 μl of DMSO was added to each well, and the plate was shaken for 10 minutes. Finally, the absorbance was measured using a standard spectrophotometer within 490 nm in triplicate. The cell inhibition was calculated based on the data for each day and each dose.

*Western blot assay*

After the cells were administered GAs for 24 hours, the medium was removed, and the cells were washed three times with ice-cold phosphate-buffered saline (PBS; pH 7.4). The cells were subsequently harvested with RIPA buffer (2% Nonidet P-40, 0.2% sodium dodecyl sulfate (SDS), 50 mM Tris buffer (pH 7.4), and 150 mM sodium chloride) mixed with a protease inhibitor cocktail and a phosphatase inhibitor cocktail for 30 min at 4°C. The concentrations of the samples were quantified by a BCA kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. Equivalent amounts of protein from each sample were loaded onto an 8-12% polyacrylamide gel for electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, CA, USA). The membrane was blocked with TBST with 5% BSA for 1 hour. The membrane was incubated with various primary antibodies for 2 hours at 37°C. After washing with TBST for 30 min * 3 times, the HRP-conjugated secondary antibodies were incubated at room temperature for 1 h. After washing as described above, immunoblots were detected with the ECL Detection System.

*RNA interference with siRNA*

When the HGC cells in 6-well plates were approximately 50-70% confluent, CHOP-specific siRNA was administered to the cells at a concentration of 50 nM following the manufacturer's instructions for 48 h. Then, the cells were treated with GAs as described above for 24 h, and the cells were harvested and analyzed by Western blotting.

*Immunofluorescence*

After C15 and C17 treatment, the cells were fixed with 4% paraformaldehyde for 24 h at 4°C, washed with PBS 3 times for 5 min, permeabilized using 0.3% Triton X-100 (PBS; pH 7.4) for 10 min, washed with PBS as described above, and then detected with primary antibodies against GRP78 and secondary 488-conjugated antibodies. The cells were then monitored by immunofluorescence microscopy.

*Transmission electron microscopy*

After administration of GAs, cells were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) at room temperature for 1 h, and transmission electron microscopy (TEM) was carried out to monitor the ultrastructure of the cells. Thin sections were cut and examined at 200 kV under an H-600 transmission electron microscope.

*TUNEL*

After administration of GAs for 24 hours, the cells were fixed with 4% paraformaldehyde overnight at 4°C, stained according to the TUNEL Kit manufacturer's instructions, and monitored by a microscope.

TUNEL Kit Protocol: 1. The adherent cells preparation: after administration of GAs for 24 hours, the cells were harvested; 2. Fixation and block: after being fixed with 4% paraformaldehyde overnight at 4°C, we added the 3% BAS (BSA in PBS) at 37°C for 60 min; 3. Permeabilisation of samples: the cells were given 0.1% Triton X-100 (Triton X-100 in PBS), 2 min at 4°C; 4. Addition of the tunel reaction mixture: we added 50 ul TUNEL reaction mixture to the sample for 60 min at 37°C; 5. Addition of Converter-POD: we added 50 ul Converter-POD on the sample for 30 min at 37°C; 6. Addition of substrate solution: we added 50-100 ul DAB substrate for 10 min at 25°C; Between every step, the sample was washed with PBS 3 times.

## Results

*Both C15 and C17 inhibited HGC cells in a time-dependent and dose-dependent manner*

The HGC cell inhibition curves of C15 and C17 were monitored by MTT. After treatment with various concentrations of C15 or C17 for various times, the cells were assessed by MTT assay. The viability of HGC cells decreased as the concentration or culture time increased for

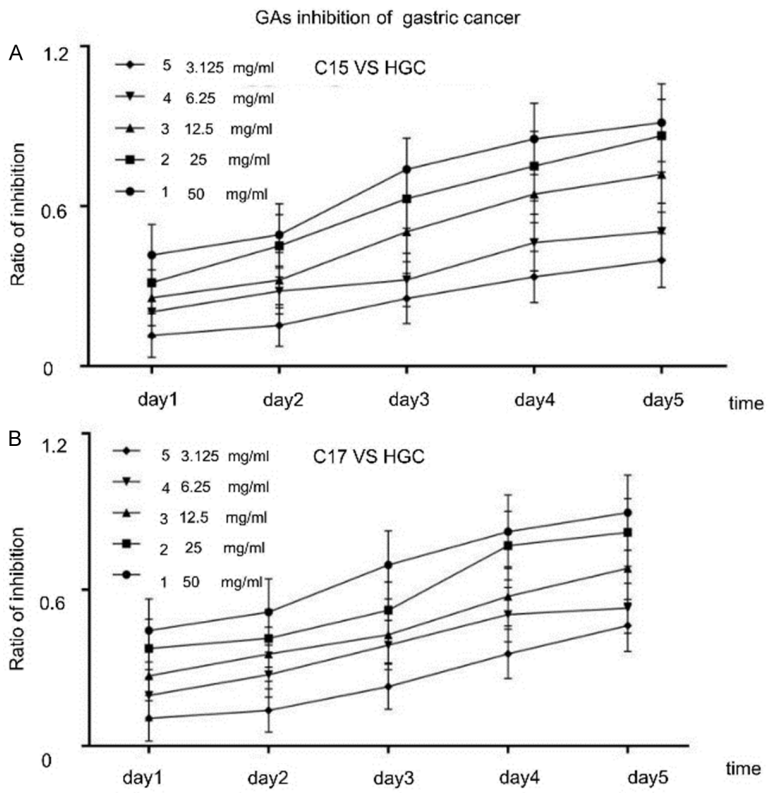


**Figure 1.** The proliferation changes of HGC cells after GA (C15 and C17) treatment. A and B. Ratio of the inhibition curve of HGC cells after GA treatment at various concentrations at different time points (n = 5 days). The ratio of inhibition with GAs (C15 and C17) showed a significant increase ($P < 0.05$) as the time or concentration increased. There was no significant difference between C15 and C17 at the same time and same concentration.

both C15 and C17. As the concentration increased, the OD of MTT for both C15 and C17 decreased in HGC cells at the same culture time. The inhibition of C15 and C17 of HGC was not significantly different at the same concentration. The inhibition ratios of HGC cells by C15 and C17 at various times and concentrations are shown in **Figure 1**. According to the formula IC50 = lg - 1[Xm-i(ΣP - 0.5)] (where Xm denotes the log of the maximum concentration of the experiment, i represents the log of each concentration measured in the experiment, ΣP represents the sum of the ratio of inhibition of each group and 0.5 is an empirical constant), the IC50 values of C15 and C17 were all calculated to be approximately 25 μg/ml.

*Both C15 and C17 induce ER stress in HGC cells*

We monitored ER stress-related protein expression after treatment with 25 μg/ml C15 and C17 by Western blotting, and GRP78 and CHOP expression was increased after treatment with C15 or C17 for 24 h in HGC cells compared with the control group. However, the expression of the proteins in C15 and C17 was not significantly different. In addition, the ER stress-related proteins eif2α, p-eif2α, ATF6, and XBP1 were also monitored by WB, which showed an increase in eIF2α phosphorylation after C15- or C17-treated HGC cells (shown in **Figure 2**).

When cells were stained for two ER stress-specific markers, GRP78 and CHOP, we observed that GRP78 and CHOP protein expression was markedly upregulated in the C15 or C17 groups compared with the control group (shown in **Figure 3**).

*C15- and C17-introduced ER stress contributes to HGC autophagy and apoptosis*

The above study showed that autophagy and apoptosis occurred in C15- and C17-treated HGC cells. Additionally, many papers have reported that ER stress can induce autophagy. We investigated whether the ER stress response induced by C15 and C17 in HGC cells has crosstalk with autophagy and apoptosis. We monitored beclin-1 and LC3, an autophagy marker protein, and caspase-3, an apoptosis-related protein, with or without 4-PBA pretreatment. 4-PBA is a chemical chaperone that is known to stabilize protein conformations to decrease the ER stress response. As shown in **Figure 4**, beclin-1, LC3-II/I and caspase-3 expression were downregulated compared with the no 4-PBA group. As a result, C15 and C17 can increase autophagy and apoptosis by increasing the ER stress response.

The TUNEL assay was also used to monitor apoptosis in HGC cells after C15 and C15 with 4-PBA treatment. The level of apoptosis in HGC

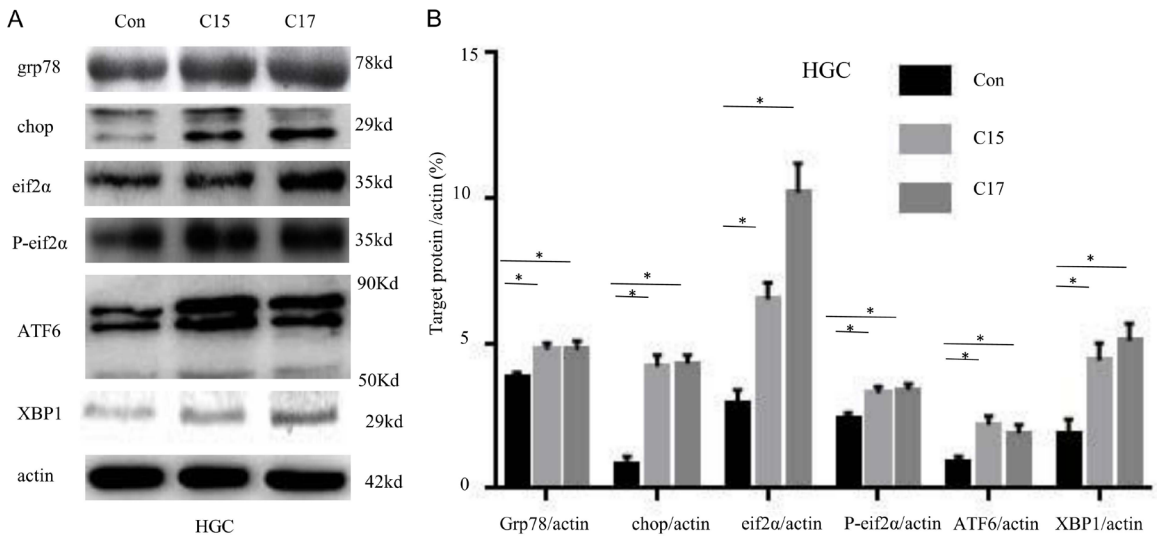


**Figure 2.** C15 and C17 induce ER stress-related protein expression in HGC cells. A and B. HGC cells were treated with C15 or C17 for 24 h. C15 or C17 increased GRP78, CHOP, eif2α, p-eif2α, ATF6, and XBP1 in HGC cells; however, there was no significant difference between the C15 and C17 groups (*, $P < 0.05$).

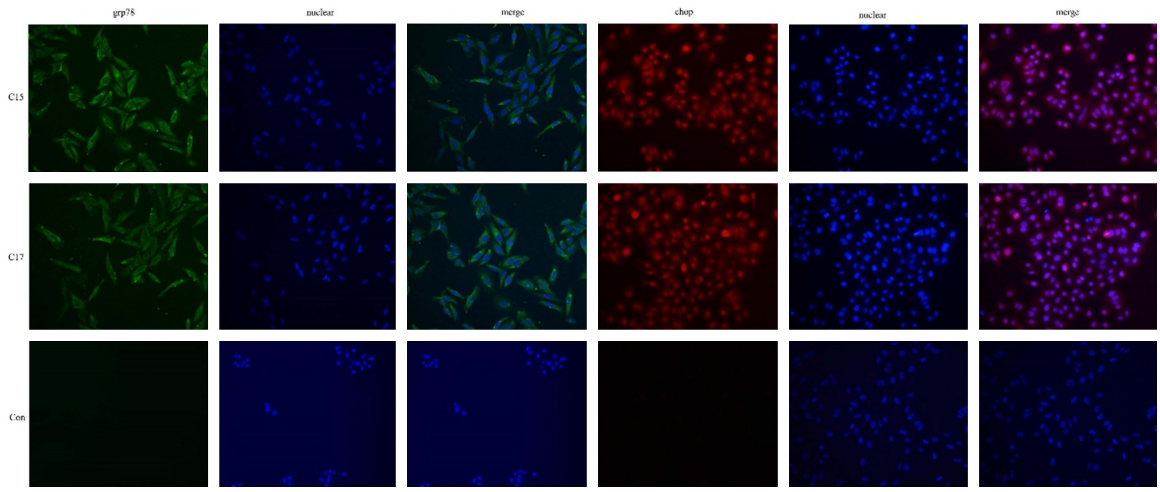


**Figure 3.** C15- or C17-treated HGC cell ER stress-related protein expression. Hoechst 33342 stained the nuclei, and the stained cells were monitored by immunofluorescence microscopy (×200 magnification). GRP78 and CHOP protein expression was much higher in the C15 and C17 groups than in the control group, and there was no significant difference between the C15 and C17 groups.

of the C15-treated groups was markedly increased compared with that of the control group and C15+4-PBA group, as shown in **Figure 5**.

*CHOP plays an important role in C15 and C17 and contributes to the ER stress response and autophagy*

When the UPR increased, the CHOP promoter was positively regulated to increase autophagy marker proteins and caspase protein expression. We silenced CHOP with specific siRNA (shown in **Table 1**) following the manufacturer's instructions, and beclin-1 and LC3 protein expression induced by C15 and C17 was also reduced by CHOP-specific siRNA. In parallel, caspase-3 expression was also decreased. The results show that the ER stress-mediated autophagy and apoptosis induced by C15 and C17 may occur via the CHOP pathway, as shown in **Figure 6**.

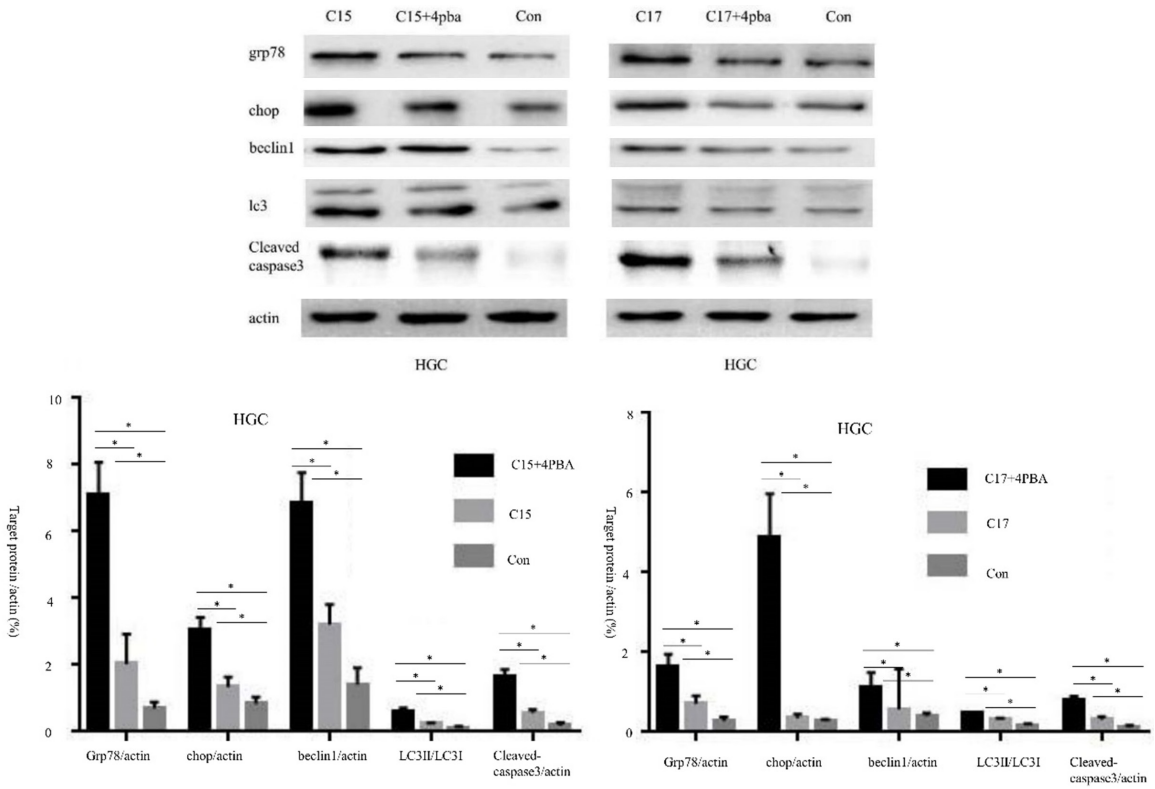


**Figure 4.** The autophagy- and apoptosis-associated proteins were downregulated in the C15 of 17 treated HGC. Beclin-1 and LC3-II/I decreased in cells in the 4-PBA+C15 or 4-PBA+C17 group compared with cells treated only with C15 or C17 without 4-PBA (*, $P < 0.05$).

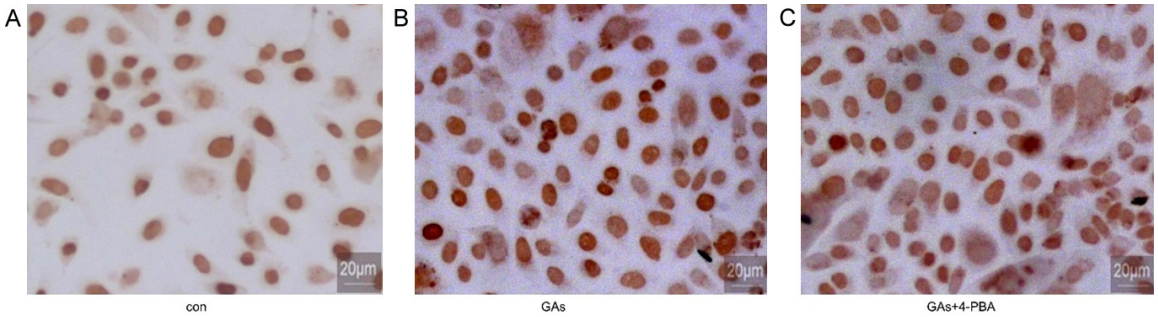


**Figure 5.** Images of TUNEL staining of HGC cells treated with C15 for 24 h (200×, magnification). The same was observed for apoptosis-associated proteins. (A-C) for microscopy: (A) control group, (B) GA group, (C) GA+4PBA group. Apoptosis of GA-treated cells was obvious compared with the control and GA+4PBA groups.

**Table 1.** Sequence interference of CHOP gene

| Name | | Sequence | |
|---|---|---|---|
| Chop (GADD153) | SiRNA1 | Sense | 5' GAGCUCUGAUUGACCGAAUTT 3' |
| | | Antisense | 5' AUUCGGUCAAUCAGAGCUCTT 3' |
| | SiRNA2 | Sense | 5' CCCAUUAUCCUGCAGAUGUTT 3' |
| | | Antisense | 5' ACAUCUGCAGGAUAAUGGGTT 3' |
| | SiRNA3 | Sense | 5' CGGAAACAGAGUGGUCAUUTT 3' |
| | | Antisense | 5' AAUGACCACUCUGUUUCCGTT 3' |

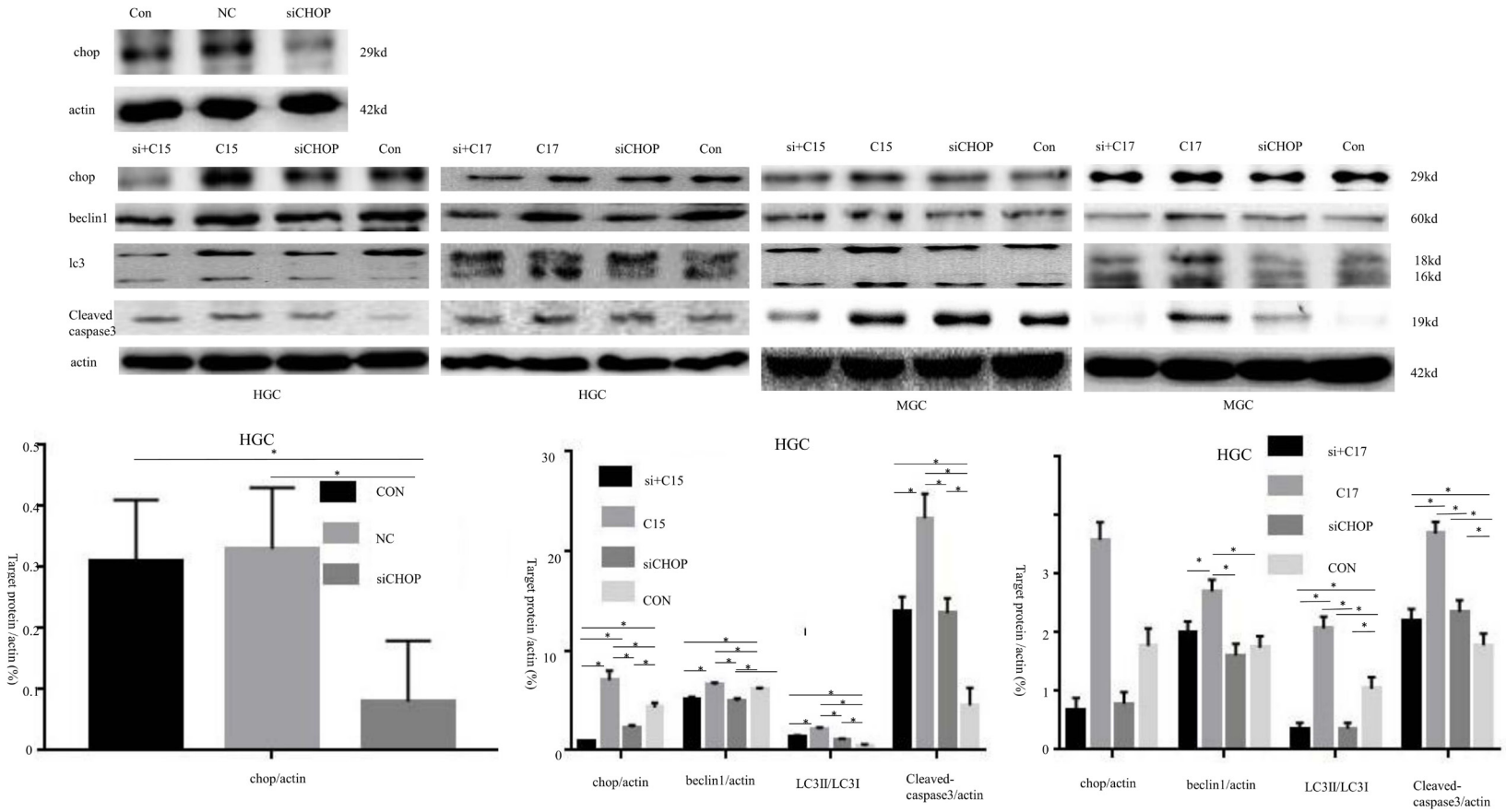


**Figure 6.** si+C15: siCHOP+C15; si+C17: siCHOP+C17. Autophagy-related and apoptosis-related protein expression decreased after CHOP was knocked down. CHOP protein expression was significantly silenced by siRNA administration. Beclin-1 and LC3-II/I expression decreased in cells after silencing CHOP in the C15 and C17 groups compared with the C15 and C17 groups without siRNA treatment. The same was observed for apoptosis-associated protein (*, $P < 0.05$).

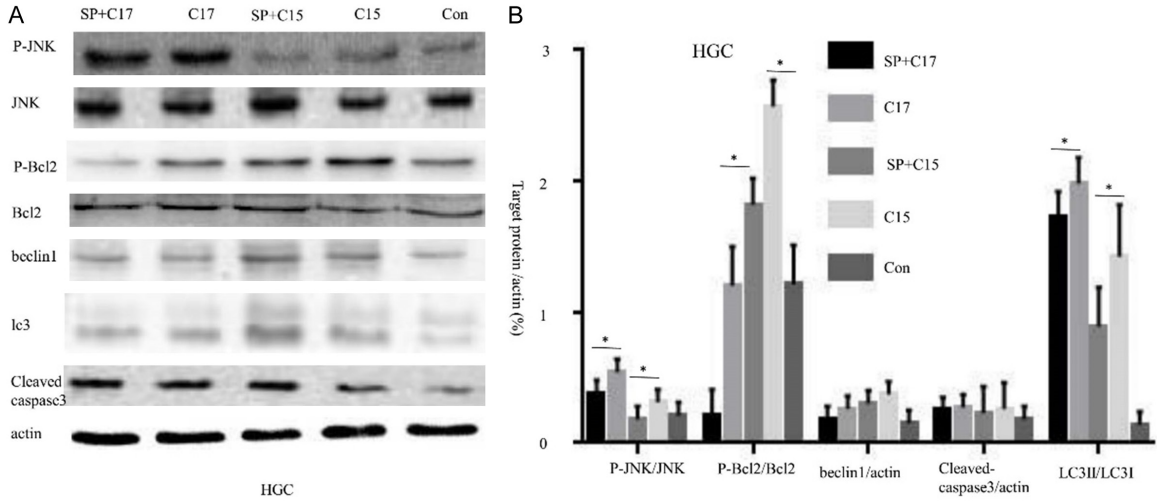


**Figure 7.** SP+C15: SP600125+C15; SP+C17: SP600125+C17. C15- and C17-induced autophagy was regulated by the IRE1/JNK/beclin-1 signaling pathway. A and B. The phosphorylation of JNK and bcl-2 expression was increased in C15- and C17-treated HGC cells. Beclin-1 and LC3-II/I expression was decreased in cells after C15 and C17 treatment with SP600125, a specific inhibitor of JNK, compared with cells treated only with C15 and C17 (*, $P < 0.05$).

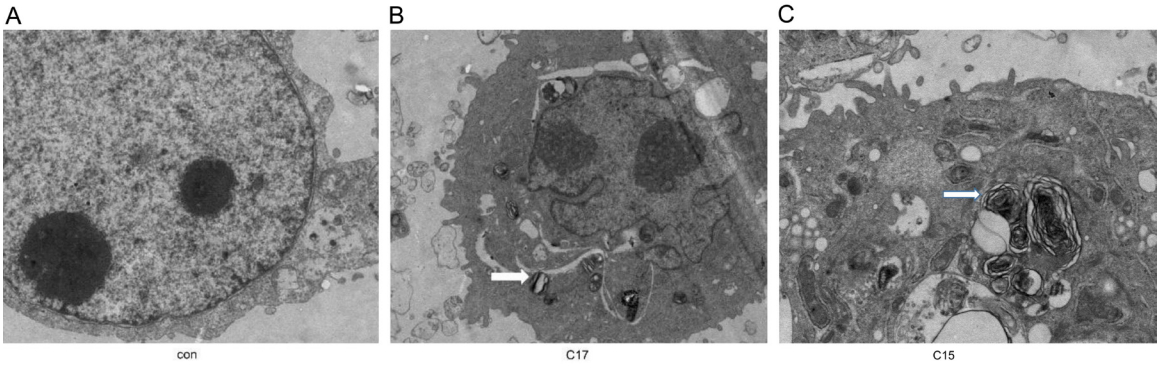


**Figure 8.** The ultrastructure of HGC cells after GA application. (A) For the control group, (B) for the C15 group, and (C) for the C17 group. The white arrow indicates autophagosomes.

*The IRE1/JNK/beclin-1 pathway participates in C15 and C17-induced autophagy*

It has been reported that the balance level of the bcl-2/beclin-1 complex regulates autophagy. When ER stress is activated, JNK can disrupt the balance by phosphorylating the anti-apoptotic protein bcl-2. To determine whether the IRE1/JNK/beclin-1 pathway participates in C15 and C17-induced autophagy, we detected p-JNK and beclin-1 exposure in C15 and C17 cells pretreated with or without SP600125, a specific inhibitor of JNK, for 2 hours. As shown in **Figure 7**, bcl-2 and beclin-1 phosphorylation was decreased, and the conversion of LC3-I was significantly decreased at the same time. It is thought that the IRE1/JNK/beclin-1 pathway participates in C15 and C17-contributed autophagy. The ultrastructure of HGC cells after GA application increased the number of autophagosomes, as shown in **Figure 8**.

*Autophagy plays a role in ER stress-mediated apoptosis*

A previous study showed that C15 and C17 contributed to HGC cell autophagy. Here, we detected GRP78, CHOP and p-eIF2α as ER stress markers. As shown in **Figure 7**, when C15 and C17 contributed to autophagy inhibition with 3MA, an apoptosis inhibitor, the expression of ER stress-associated proteins was also decreased, as shown in **Figure 9**. These results indicate that both autophagy and

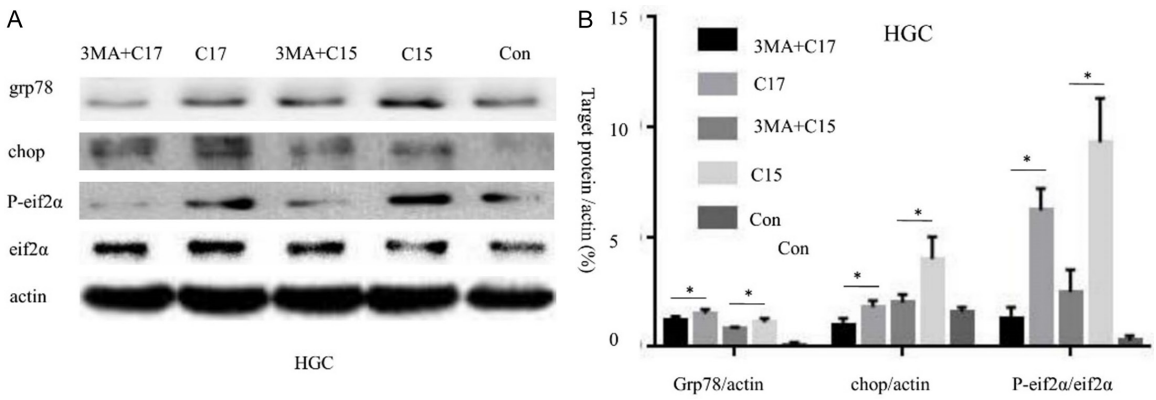


**Figure 9.** The expression of ER stress-related proteins in HGC cells after downregulated autophagy, introduced by C15 and C17 application, with 3MA. A and B. GRP78, CHOP and p-eIF2α expression was decreased in cells after 3MA treatment compared with cells without 3MA treatment (*, $P < 0.05$).

autophagy induced by C15 and C17 are pro-apoptotic mechanisms and that downregulated autophagy reduces C15- and C17-induced ER stress and apoptosis.

## Discussion

GAs have been used extensively as cardiovascular protective agents in both preclinical and clinical studies [22]. Recently, an increasing number of studies have focused on its antitumor activity [23]. Previous reports have demonstrated that mixed natural Ginkgolic acids have antitumor potency [11, 24], but which of the mixed compounds is most powerful remains unclear. Multiple mechanisms underlying GA cytotoxicity have been elucidated, including immunomodulation, autophagy pathways, and apoptotic pathways [25, 26]. Additionally, several reports have indicated possible crosstalk among these mechanisms.

It has been reported that GAs can inhibit sumoylation by inhibiting the SUMO activating enzyme E1, and the lowest binding energies of GA on SUMO E1 were determined by AutoDock4 using the Lamarckian algorithm [27]. All seven GAs showed high binding affinities, and all compounds bound to the same pharmacophore. Additionally, C15 and C17 have similar binding energies [28], and C15 and C17 have similar inhibition curves on HGC.

Our study clearly confirmed for the first time that the ER stress response is involved in gastric carcinoma cells treated with ginkgolic acid. This conclusion was supported by the above findings. First, the ultrastructure of HGC treated with C15 or C17 showed dilatations of ER cisternae in morphology [29]. Then, GRP78/BiP molecular chaperone expression was increased significantly in HGC cells after application of C15 or C17. GRP78 can prevent misfolding during translocation by changing the hydrophobic domains of proteins [30]. The misfolding protein initiates the UPR [31], so with GRP78 depletion, the UPR is widely activated, and the ER stress response follows excessive UPR [32]. Third, ER stress has three parallel signaling pathways [32], named IRE1, ATF6, and PERK. The expression of each pathway marker, PERK, phosphorylated eIF2α and XBP-1, was increased. Finally, after applying 4-PBA to reduce ER stress, the autophagy and apoptosis induced by C15 and C17 were also suppressed, and in addition, after specific siRNA-mediated downregulation of CHOP, LC3-II conversion and caspase-3 expression were attenuated. All the findings suggest that ER stress led to autophagy and apoptosis in C15- and C17-treated HGC cells. To the best of our knowledge, all these data indicate that the autophagy, apoptosis and ER stress introduced by C15 and C17 must have crosstalk.

It has been reported that there are two potential pathways by which ER stress triggers autophagy [33, 34]: the PERK/eIF2α and IRE1/JNK pathways. First, IRE1/JNK can release beclin-1, and beclin-1 triggers autophagy by autophagosome formation and LC3-II conversion [35]. The beclin-1 and bcl-2 proteins both have the BH-3 domain, so beclin-1 inhibition can be alleviated by bcl-2 regulation, and the

balance of autophagy and apoptosis is regulated [36, 37]. p-JNK and beclin-1 expression was increased after HGC and MGC were administered C15 and C17. After pretreatment with a JNK-specific inhibitor named SP600125, p-JNK, p-bcl-2, and beclin-1 and the conversion of LC3-I were downregulated. Second, when the UPR is increased, CHOP must be upregulated through the PERK/eIF2α pathway, and the CHOP protein can provoke caspase activation [38]. In this paper, after C15 and C17 were applied, CHOP expression in HGC cells was increased, and after CHOP-specific siRNA suppressed CHOP expression, both caspase-3 and LC3-I conversion and beclin-1 were suppressed. We propose that the increased CHOP in HGC cells mediates autophagy and apoptosis induced by C15 and C17. We suggest that ginkgolic acid-induced ER stress triggers autophagy and that the IRE1/JNK/beclin-1 and PERK/eIF2α/CHOP pathways participate in this process.

It has been reported that excess ER stress can cause autophagy, and whether autophagy is cytotoxic or cytoprotective is still unclear [39, 40]. In this paper, after autophagy induced by C15 and C17 was inhibited with 3MA, GRP78, CHOP and p-eIF2α expression was also decreased. It has been reported that time is the key for whether autophagy is cytotoxic or cytoprotective [41, 42]. At an early stage, autophagy can attenuate anticancer therapy cytotoxicity, making it cytoprotective, while at a late stage, autophagy can augment the cytotoxicity of anticancer therapy. In this paper, the ER stress and apoptosis induced by C15 and C17 were relieved after administration of 3MA.

In conclusion, this paper suggests that the UPR signaling pathways take part in HGC cells affected by C15 and C17, and all three branches of the UPR are involved. In addition, autophagy plays a central role in ginkgolic acid-induced apoptosis, since ginkgolic acids activate the PERK/eIF2α/CHOP and IRE1/JNK/beclin-1 pathways, and these two pathways introduce autophagy, while inhibiting autophagy and relieving ER stress and apoptosis induced by ginkgolic acids. Overall, this paper suggests that ginkgolic acid-induced ER stress and autophagy mediate apoptosis, making it a powerful antitumor candidate.

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## Disclosure of conflict of interest

None.

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