INTRODUCTION

*Cinnamomum cassia* (called Chinese cassia or Chinese cinnamon) is an evergreen tree that is widely found in southern and eastern Asia [1]. Cinnamaldehyde (CA) is one of the most important bioactive ingredients in *Cinnamomum cassia*, and is often used as traditional Chinese medicine [2]. CA has diverse biological functions including anti-oxidant, anti-inflammation, anti-diabetic, and anti-cancer effects [3–6]. CA is a natural flavonoid, and flavonoids like luteolin, quercetin, and CA are powerful natural anti-cancer compounds that function via the inhibition of histone deacetylase (HDAC) [7–9]. Emerging HDAC inhibitors that induce powerful apoptosis and cell death in various cancer cell types have emerged as potent anticancer agents that can restore programmed cell death in malignant cells [10]. HDACs are upregulated in many cancer cells, and HDAC inhibition regulates cell death or growth-related genes via acetylation and methylation of histones [11]. HDAC8 has been studied to play a potential role for tumor growth and proliferation, whereas HDAC8 inhibition caused apoptosis, cell death, and cell cycle arrest in various cancer types [12].

Histone methylations via epigenetic modification on lysine 4, 9, and 27 of H3 play a potential role as cell survival or death codes and perform transcriptional activator and inhibitor activities for histone-bound genes [13]. HDAC inhibitors can frequently suppress histone methyl transferases, such as G9a and EZH2, and their inhibition regulates methylation on lysine 9 and 27 of H3 [14]. G9a is reportedly involved in cell development, cell differentiation, cell cycle, and autophagic cell death [15, 16]. G9a normally binds on LC3B promoter, but repressive binding complex with RFX5, Sin3B, and HDACs binds on LC3B promoter and blocks LC3B expression [17, 18]. Targeting HDAC regulates autophagy-related genes by inhibiting G9a [19]. BIX01294, selective inhibitor of G9a/EHMT2, could powerfully mediate autophagy and apoptosis through the mTOR/AMPK/ULK1 pathway in various cancer types, such as gastric, breast, oral squamous carcinoma, glioma, and neuroblastoma [20–22]. G9a inhibition may be a potential anti-cancer therapeutic strategy and a promising tumor therapeutic target.

Endoplasmic reticulum (ER) has potential function in protein translocation, folding, and maturation [23]. Disruption of ER function by diverse stimuli and pathological conditions induces ER stress by increasing misfolded or unfolded proteins [24]. Emerging reports suggested that severe or excessive ER stress causes cell death in various diseases via powerful, anticancer-related molecular mechanisms [25]. In particular, sustained intracellular calcium (Ca**{2+}**) release in ER lumen resulted in ER stress-induced cell death via Ca**{2+}** interacting chaperones such as GRP78/Bip [26]. ER stress regulates diverse signaling pathways via the activation of three ubiquitous branches; PRKR-like ER kinase (PERK), inositol-requiring enzyme1 alpha (IRE1α), and activating transcription factor 6 (ATF6) [27]. In normal conditions, GRP78/Bip binds to unfolded protein response (UPR) such as PERK and IRE1α and inhibits ER stress signaling. However, under ER stress, GRP78 dissociates with UPR sensors [28]. With PERK release by ER stress, PERK phosphorylates eukaryotic translation initiation factor-2α (eIF2α), and its phosphorylation stimulates the activation of activating transcription factor-4 (ATF4) in the cytosol and -CCAAT/-enhancer-binding protein homologous protein (CHOP)
LDH assay

Cells (1 × 10^5 cells/well) were seeded into a 96-well plate with RPMI-1640 growth medium. To determine the LDH (Thermo Scientific Pierce; 13464269) activity in supernatants, 100 μL of the reaction mixture was added and incubated for 30 min in a dark room at room temperature. The LDH activity was measured by the absorbance of the samples at 490 or 492 nm using a microplate reader (Molecular Devices, USA).

Transfection

NCI-N87 and MKN-74 cells (3 × 10^5 cell/well) were transfected with double-stranded siRNAs (30 nmol/mL) of LC3B (Santacruz; sc-43390), ATG5 (Santacruz; sc-41445), PERK (Santacruz; sc-36213), G9a (Santacruz; sc-43777), and CHOP (Bioneer; 1649-1) in a six-well plate for 24 h by the Lipofectamine 2000 (Invitrogen; 11668019) method according to the manufacturer’s protocol and were then recovered in RPMI-1640 medium (Welgene) containing 5% fetal bovine serum (Gibco) and 100 μg/mL antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Gibco) for 24 h. After recovering, viable cells were measured and analyzed using the WST-1 assay.

Isolation of protein

Protein cell lysates from cells (2 × 10^6 cell/well) in a 100-mm cell culture dish were collected in RIPA buffer containing a protease inhibitor cocktail (Sigma; P8340) on ice for 30 min. And then Protein cell lysates were passed through an 18-gauge needle and spun down. The supernatant was analyzed for protein content using the BCA method (Thermo scientific, Pierce BCA Protein Assay Kit, USA; 23225).

Western analysis

For Western blotting analyses, cells were solubilized in radioimmunoprecipitation assay (RIPA) lysis buffer (50-mM Tris-HCl (pH 7.4), 150-mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1-mM phenylmethylsulfonyl fluoride (PMSF), 1-mM sodium orthovanadate, and 1× Sigma protease inhibitor cocktail), and protein content was measured using a standard bicinchoninic acid assay. Equal amounts of protein (20 μg) were size-fractionated by 8%–15% SDS-PAGE and then transferred onto a PVDF membrane (Millipore Corporation, Billerica, MA, USA; IPVH00010). Membranes were blocked by incubation for 30 min with 5% skim milk/PBS-T (PBS with 5% powdered milk and 1% Tween20 (Sigma; P9416)) and incubated overnight at 4 °C with primary antibodies diluted in 1× PBST buffer. The following primary antibodies were used: β-actin (sc-47778), Bcl-2 (sc-7382), Beclin-1 (sc-48341), ULK1 (sc-390904), GRP78 (sc-166490), eIF2α (sc-331312), and ATG5 (sc-131518) (Santa Cruz; 1:1000); LC3B (Sigma, 1:1000; L7543); p62 (Sigma, 1:1000; P0068); G9a (Abcam, 1:1000; ab40542); and cleaved caspase-3 (#9654), cleaved caspase-9 (#9505), p-AMPK (Thr172; #50082), AMPKα (sc2793), p-mTOR (Ser2448; #2796), mTOR (sc2796), p-TOR (Ser2448; #5356), and Akt (sc2922) activity in supernatants, 100 μL of WST-1 reagent added to each well of a 96-well plate (1 × 10^5 cell/well). To determine the cell viability in the supernatant, the conversion of WST-1 reagent into chromatographic formazan was measured and analyzed with a microplate reader (Molecular devices, USA).
Confocal microscopy was performed using a ZEISS LSM5 PASCAL confocal microscope with 405 and 488-nm excitation lasers.

Immunoprecipitation (IP) assay
We extracted cell lysates from NCI-N87 and MKN-74 cells (2 × 10⁶/well) on a 100-mm cell culture plate in an IP buffer (pH 7.5) containing 50-mM Tris-HCl, 250-mM NaCl, 5-mM EDTA, 0.5%(v/v) NP-40, and protease inhibitor cocktail (Sigma). We incubated anti-Bcl-2 (Santa Cruz) and anti-Beclin-1 (Santa Cruz) with lysate at 4 °C for 16 h. We used protein A/G plus agarose (Santa Cruz; sc-2003) to pull down immunocomplexes. We washed precipitates three times with IP buffer. We resolved the immunoprecipitated proteins with 12% SDS-PAGE and analyzed them.

Chromatin immunoprecipitation (ChIP) assay
ChIP assays were performed using an EZ ChIP Chromatin Immunoprecipitation kit (Millipore, Billerica, MA, USA; 17371) as described in the supplier's protocol. Briefly, the cross-linked chromatin was sonicated after cell lysis and then incubated overnight at 4 °C with antibodies against G9a (Abcam). The immunocomplex was precipitated with protein A-agarose (Millipore), and the beads were washed, sequentially treated with 10 µL of RNase A (37 °C for 30 min) and 75 µL of proteinase K (45 °C for 4 h) and then incubated at 65 °C overnight to reverse cross-link of the chromatin. The DNA was recovered by phenol–chloroform extraction and co-precipitation with glycogen and was then dissolved in 50 µL of Tris-EDTA (TE) buffer. DNA associated with the ER was amplified by PCR using 1 µL of precipitated DNA. PCR primers (5′-GAATTGCTATGCCTACTA-3′ (sense) and 5′-GCCGTCGTCTCGTTCTCC-3′ (antisense)) were designed to amplify the G9a binding site on the LC3B gene promoter and PCR primers (5′-CCCGTATACCATCCTTAGTCC-3′ (sense) and 5′-GAAACTCGTGTCCAGTTCAG-3′ (antisense)) were designed to amplify the G9a binding site on the Beclin-1 gene promoter. Quantitative PCR conditions were 40 cycles at 94 °C for 40 s, 60 °C for 1 min, and 72 °C for 40 s.

Measurement of intracellular Ca²⁺ level
Calcium release assays were performed using a Calcium Assay Kit (Coloriometric) (Abcam; ab102505) as described in the supplier's protocol. For the calcium release experiments, NCI-N87 and MKN-74 cells (1 × 10⁵ cells/well) were seeded and incubated into a 96-well plate with growth medium. Then, the cells were treated with CA, NCI-N87 and MKN-74 cells were washed with calcium-free buffer and then added to chromogenic reagent and calcium assay buffer for 10 min at room temperature. The fluorescence was measured and analyzed by the absorbance of the samples at 575 nm using a microplate reader (Molecular Devices, USA).

Statistical Analysis
All results were confirmed in at least three independent experiments; Student's t-tests were used for between-groups comparisons of the means of quantitative data, and P < 0.05 was considered statistically significant.

RESULTS
CA suppresses the growth of GC cells
To identify the cytotoxic effects of CA in GC, we performed the cell viability and LDH release using WST-1 assay and LDH assay at indicated doses. We found that CA triggers anti-proliferative effects through a dose-dependent decrease of cell viability and increase of LDH production compared with control in various GC cell types, including SNU-638, SNU-216, AGS, NCI-N87, MKN-45, and MKN-74 cells (Fig. 1a, b). In addition, it was found that the anti-proliferative effects of CA on NCI-N87 and MKN-74 cells were time-dependent and that CA mediates LDH cytotoxicity in GC cells (Fig. 1c, d). The cytotoxic effect was identified by the increase of caspase 3 and 9 cleavage and downregulation of Bcl-2 in NCI-N87 and MKN-74 cells (Fig. 1e). To evaluate whether CA-treated cytotoxicity was associated with apoptosis, we co-treated NCI-N87 and MKN-74 cells with CA and Z-VAD-FMK (50 µM), a pan-caspase inhibitor. Our results indicated that Z-VAD-FMK alone did not significantly change the cell viability and LDH release but CA induced the decrease of cell viability and increase of LDH cytotoxicity in NCI-N87 and MKN-74 cells; however, Z-VAD-FMK inhibits the effects of CA on cell viability and LDH cytotoxicity GC cells (Fig. 1f, g). Furthermore, to further identify relationship between CA and apoptosis, we performed Western blotting analyses. Z-VAD-FMK treatment inhibits caspase-3 cleavage and CA alone mediates caspase-3 cleavage in CA-induced GC cells; however, Z-VAD-FMK suppresses caspase-3 cleavage in CA-treated GC cells (Fig. 1h). These results indicated that CA causes apoptotic cell death in GC cells.

CA mediates autophagy in GC cells
To identify whether CA regulates autophagy in GC cell, we performed Western blotting analysis which would determine the expression of the autophagy-related genes, LC3B and p62. It was found that CA causes dose-dependent increase of LC3-II and decrease of p62 in NCI-N87 and MKN-74 cells (Fig. 2a). To further analyze the autophagy effects of CA in NCI-N87 and MKN-74 cells, Western blotting analysis was performed. These results showed that CA treatment powerfully downregulated the expression of p62 and upregulated the expression of ATG5, Beclin-1 and LC3-II (Fig. 2b). To confirm autophagic vacuoles by CA treatment, pEGFP-LC3 vector was transiently transfected into both NCI-N87 and MKN-74 cells. Control cells have low LC3 puncta, whereas NCI-N87 and MKN-74 cells by CA treatment have high LC3 puncta (Fig. 2c). Anti-apoptotic Bcl-2 interacts with the autophagy-related protein Beclin-1 in ER lumen, and both Bcl-2 and Beclin-1 dissociate with the initiation of the autophagy process [41]. To investigate whether Bcl-2 interacts or dissociates with Beclin-1 in CA-treated GC cells, we performed co-immunoprecipitation (Co-IP) assay in CA-treated GC cells. When we performed the Co-IP assay with Bcl-2 antibody, Bcl-2 bound with Beclin-1 in GC cells, whereas CA treatment suppressed the interaction between Bcl-2 and Beclin-1. Furthermore, in the Co-IP assay with Beclin-1 antibody using lysates from NCI-N87 and MKN-74 cells, Beclin-1–Bcl-2 interaction was also disturbed by CA treatment (Fig. 2d). Therefore, CA treatment in GC cells leads to autophagy activation via the interruption of the Bcl-2/Beclin-1 complex. To identify synergic autophagy effects of the combination of CA with cisplatin (5 µM) or paclitaxel (50 nM) in GC cells, we evaluated cell viability, LDH release, and the expression of autophagy-related genes such as p62, ATG5, and LC3B. Consequently, compared to CA alone, both CA + cisplatin and CA + paclitaxel decreased cell viability, enhanced LDH release, and induced the autophagy process, such as the upregulation of ATG5 and LC3B and the decrease of p62 expression (Fig. 2e and f). Taken together, our findings indicate that CA + cisplatin or CA + paclitaxel treatments induce synergic autophagic cell death in GC cells.
Cell viability and LDH cytotoxicity induced by cinnamaldehyde (CA) in GC cells, including SNU-638, SNU-216, AGS, NCI-N87, MKN-45, and MKN-74, measured using WST-1 and LDH assays in a dose-dependent manner (0, 25, 50, and 100 μg/mL; 24 h) and a time-dependent manner (0, 8, 16, and 24 h). Cell viability and LDH release of control cells were set at 100%; *P < 0.05. Western blotting analyses of cleaved caspase-3 and -9 analyzed on the indicated times (0, 8, 16, and 24 h; 50 μg/mL) CA-treated NCI-N87 and MKN-74 cells; *P < 0.05. f-h Effect of Z-VAD-FMK (50 μM) on CA-induced apoptotic cell death. NCI-N87 and MKN-74 cells were pretreated with Z-VAD-FMK for 4 h and were subsequently treated with CA (50 μg/mL, 24 h). Cell viability was determined using the WST-1 assay, and cell cytotoxicity was analyzed using the LDH assay; *P < 0.05. Total protein samples were analyzed by Western blotting analyses using apoptosis markers such as cleaved caspase-3 and -9. β-actin was used as a protein loading control.

Fig. 1 Anti-cancer effects of cinnamaldehyde in GC cells. a-d Cell viability and LDH cytotoxicity induced by cinnamaldehyde (CA) in GC cells, viability analyses, the LDH assay, and Western blotting analysis. It was found that both 3-MA and CQ induce the restoration of cell viability and the inhibition of LDH release in CA-treated GC cells (Fig. 3a, b). In Western blotting analysis, 3-MA inhibited the expression of LC3-II indicating the disturbance of autophagosome formation, whereas both CA and CQ mediated the accumulation of LC3-II, indicating the disturbance of autophagosome formation and the interruption of the fusion of autophagosomes with lysosomes (Fig. 3c). 3-MA decreased LC3B expression in CA-treated NCI-N87 and MKN-74 cells, whereas CQ exerted the accumulation of LC3-II (Fig. 3c). Our result suggested that CA modulates the autophagic flux in GC.

Autophagy knockdown inhibits CA-induced cell death in GC cells To confirm the knockdown of autophagy-related genes, NCI-N87 and MKN-74 cells were transfected with ATG5- and LC3B-specific siRNA, respectively, and cultured with CA treatment for 24 h; then they were subjected to cell viability analyses, the LDH assay, and Western blotting analysis. The effects of CA on cell viability and LDH release were reversed in ATG5 and LC3B knockdown NCI-N87 and MKN-74 cells compared to CTL knockdown cells (Fig. 3d, e, g, h). Furthermore, the upregulation effects on ATG5 and LC3B expression by CA were inhibited in ATG5 and LC3B knockdown NCI-N87 and MKN-74 cells (Fig. 3f, i). These results indicate that targeting autophagy disturbs autophagic cell death in CA-treated NCI-N87 and MKN-74 cells.

mTOR-AMPKa-ULK1 modulates cinnamaldehyde-mediated autophagic cell death in GC cells To investigate the hypotheses regarding CA-induced autophagic cell death, the mTOR–AMPK–ULK1 axis in CA-treated NCI-N87 and MKN-74 cells was identified using Western blotting analysis. We demonstrated that the expression of p-AMPKα and p-ULK1 was significantly enhanced in a time-dependent manner, whereas the expression p-mTOR was downregulated by CA treatment at the indicated times (Fig. 4a). We questioned if compound C regulates CA-induced autophagic cell death by interrupting AMPKα. We co-treated NCI-N87 and MKN-74 cells with compound C and CA and performed cell viability, LDH assay and Western blotting analysis. It was found that compound C inhibited the reduction of cell viability and LDH release in CA-induced NCI-N87 and MKN-74 cells (Fig. 4b). Compound C inhibited the expression of p-AMPKα, ULK1, and LC3B in CA-treated NCI-N87 and MKN-74 cells (Fig. 4c). Based on these findings, it was hypothesized that targeting ULK1 using a pharmacological inhibitor and specific siRNA may regulate CA-induced autophagic cell death in GC cells. SBI-0206965, a
CA induces autophagic cell death via PERK-ATF4–CHOP signaling in GC cells

Calcium (Ca^{2+}) was restored in ER lumen and differential intracellular calcium release has a functional role in understanding biological molecular mechanisms involved in ER stress-induced apoptosis and cell death, and the modification of Ca^{2+} ion concentration is a critical initiator of ER stress-related pathways such as PERK-ATF4 and IRE1α–JNK pathways [43]. To determine the changes in Ca^{2+} concentration in CA-treated GC cells, we performed the calcium assay in a time-dependent manner. The results indicated that CA treatment enhances fluorescent intensity meaning Ca^{2+} release in NCI-N87 and MKN-74 cells (Fig. 5a). These findings suggested that the increased Ca^{2+} release regulates CA-induced ER stress in GC cells. To further investigate whether ER stress is associated with CA-mediated cell death, we screened the PERK axis of the ER stress pathway in a time-dependent manner in CA-treated GC cells. CA induced ER stress in GC cells. To further investigate whether ER stress is associated with CA-mediated cell death, we screened the PERK axis of the ER stress pathway in a time-dependent manner in CA-treated GC cells. CA induced the expression of GRP78, p-PERK, p-eIF2α, and CHOP in NCI-N87 and MKN-74 cells (Fig. 5b). To investigate the effect of CA on ER stress in GC cells, we performed cell viability analysis, the LDH assay, the calcium assay, and Western blotting analyses with ER stress inducer TG and CA.
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The treatment of GCs with Cinnamaldehyde (CA) results in cell death, autophagic cell death, and ER stress-mediated cell death in CA-induced GC cells.

**Fig. 3 Targeting autophagy in cinnamaldehyde-treated GC cells.** a, b Cell viability and LDH release were analyzed using WST-1 and LDH assays in CA (50 µg/mL, 24 h)- and TG-treated NCI-N87 and MKN-74 cells with 3-MA (5 mM) or CQ (20 µM) treatment for 24 h. *P < 0.05. c Western blot analysis of LC3B in the CA (50 µg/mL, 24 h)-treated NCI-N87 and MKN-74 cells with 3-MA (5 mM) or CQ (20 µM) for 24 h. d–i After NCI-N87 and MKN-74 cells were transfected with ATG5 and LC3B siRNAs, cell viability analysis, the LDH assay, and Western blotting analyses for ATG5 and LC3B were performed in CA (50 µg/mL, 24 h)-treated NCI-N87 and MKN-74 cells. β-actin was used as a protein loading control.

PERK inhibition suppresses CA-induced autophagic cell death in GC cells

In normal conditions, PERK, a powerful ER stress sensor, exerts a suppressive effect via the interaction with GRP78 [44]. It was hypothesized that targeting PERK by specific siRNA may modulate CA-induced ER stress-mediated cell death in GC cells. After PERK and CHOP were transfected by specific-siRNA in NCI-N87 and MKN-74 cells, CA treatment was done. When we performed cell viability analysis, the LDH assay, the calcium assay and Western blotting analysis, PERK and CHOP knockdown were found to suppress the decrease of cell viability and the increase of LDH cytotoxicity and calcium release in CA-treated NCI-N87 and MKN-74 cells (Figs. 6a–c, e–g). With Western blotting analysis, PERK and CHOP knockdown led to decreased p-PERK, p-eIF2α, and CHOP expression by blocking PERK in CA-treated NCI-N87 and MKN-74 cells (Fig. 6d, h). These results suggested that PERK and CHOP knockdown suppresses ER stress-mediated cell death in CA-induced GC cells.

G9a inhibition regulates cinnamaldehyde-induced autophagic cell death in GC cells

Recent reports indicate that histone modification modulates autophagy flux and is associated with early and late stages, including H3K9me2 and H4K16 deacetylation [45]. The active ingredient aqueous cinnamon extract and its active ingredients, such as cinnamaldehyde, cinnamyl alcohol, and cinnamic acid, regulate HDAC activity [46]. BIX-01294, a G9-specific inhibitor, mediates the reduction of H3K9me2 via G9a inhibition and induces autophagic cell death through the AMPK axis [47]. To identify whether CA treatment regulates the G9a expression in GC cells, we performed the immunofluorescence assay. Our results suggested that CA inhibits the G9a expression (Supplementary Fig. 2). To validate G9a and ATF4 binding on Beclin-1 and LC3B proximal promoter in CA-treated GC cells, we performed the CHIP assay from DNA fragments by sonication. In the real-time CHIP assay, the binding sites associated with Beclin-1 and LC3B genes were bound by G9a before CA treatment in NCI-N87 and MKN-74 cells, respectively, and CA treatment inhibited G9a binding and induced ATF4 binding on Beclin-1 and LC3B promoter (Fig. 7a–c). To determine if G9a is associated with CA-mediated autophagic cell death, after NCI-N87 and MKN-74 cells were knocked down by G9a-specific siRNA, CA treatment was performed. In addition,
when we performed cell viability analysis, the LDH assay, and Western blotting analysis, cell viability was more decreased by CA in G9a knockdown cells than in control cells, and LDH release was more increased in CA-treated G9a knockdown cells than in control cells (Fig. 7d). Western blotting analysis revealed that G9a knockdown cells had upregulated LC3-II expression, and CA treatment also blocked G9a levels and increased LC3-II levels (Fig. 7e). To investigate whether co-treatment with CA and BIX-01294 is associated with autophagic cell death in CA-treated NCI-N87 and MKN-74 cells, we performed the cell viability assay, the LDH assay, and Western blotting analysis. Cell viability was significantly decreased by CA or BIX-01294 treatment, and LDH cytotoxicity was increased after CA or BIX-01294 treatment; however, CA + BIX-01294 showed further decreased cell viability and enhanced LDH release in NCI-N87 and MKN-74 cells (Fig. 7f). Furthermore, the synergistic effects of CA + BIX-01294 on cell viability and LDH release were reversed in 3-MA-pretreated NCI-N87 and MKN-74 cells (Fig. 7f). Western blotting suggested that CA + BIX-01294 downregulated the G9a expression and upregulated the LC3-II level in NCI-N87 and MKN-74 cells (Fig. 7g). In addition, 3-MA inhibited the reduction of G9a and the increase of LC3-II in CA- and/or BIX-01294-treated NCI-N87 and MKN-74 cells (Fig. 7g).

**DISCUSSION**

Many researchers have suggested that flavonoids, including kaempferol, apigenin, and quercetin, can be used as potential anti-cancer drugs owing to their potent anti-tumor effect and lesser side effects [48-50]. Although flavonoids are one of many studied phytochemicals, detailed biological experiments have encouraged to diverse disease therapeutics. Previous reports against the role of cinnamaldehyde, a flavonoid isolated from *C. cassia*, can be a novel anti-cancer drug, but there is a lack of knowledge about detailed biological and molecular mechanisms of CA. Cinnamaldehyde, a well-known HDAC inhibitor and anti-tumor reagent, exerts powerful anti-cancer effect through apoptosis and cell death in various cancer types, including colorectal cancer, non-small cell lung cancer, head and neck...
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Fig. 5 Cinnamaldehyde regulates cell death via ER stress response. a After NCI-N87 and MKN-74 cells were treated with CA (50 μg/mL) for the indicated times, intracellular calcium (Ca²⁺) assay was performed on the indicated times; *P < 0.05. b The activation of ER stress signaling, including GRP78, p-PERK, PERK, p-eIF2α, eIF2α, and CHOP was investigated using Western blotting assay. c–e NCI-N87 and MKN-74 cells were treated with TG (3 mM; 24 h) and CA (50 μg/mL, 24 h), and cell viability, LDH release, and Ca²⁺ release were determined using WST-1, LDH, and intracellular calcium assays, respectively; *P < 0.05. f PERK signaling, including p-PERK, PERK, p-eIF2α, eIF2α, and CHOP, was investigated by Western blotting analyses. β-actin was used as a protein loading control.

cancer, hepatocarcinoma, and leukemia [51, 52]. Programmed cell death type II (Autophagic cell death) was reported to have differential mechanism and signaling programmed cell death type I (Apoptosis) [53]. Unlike apoptosis, autophagic cell death induces cell death by producing ROS, losing plasma membrane integrity, and membrane oxidation degradation and then mediates cell death via the activation of many caspasers, including caspase−2, −3, −6, 8, 9, and 10 [54, 55]. Furthermore, Bcl family proteins such as Bcl-2 and Bcl-XI play a potential role to determine differential mechanism between apoptosis and autophagic cell death [56]. Bcl-2 blocks apoptosis process by inhibiting the activation of Bax and the release of cytochrome c [57]. Bcl-2 on the endoplasmic reticulum (ER) suppresses autophagy process by interacting with beclin-1 and activates autophagy process by dissociating with beclin-1 [58]. Recent reports suggested that apoptosis machinery regulates autophagy process; however, autophagy-related proteins also modulate apoptosis process [59]. In this work, we show the first evidence that CA causes autophagy and cell death via the PERK−CHOP axis of ER stress pathways and the G9a binding on Beclin-1 and LC3B promoter in GC. Cinnamomum cassia extract (CCE) could suppress human oral cancer cell growth via caspase-3 cleavage, Bcl-2 reduction, and increasing autophagic markers, including LC3A, autophagy-related protein 14, Rubicon and p62 in vivo and in vitro. However, autophagy inhibition increases apoptosis and cell death in CCE-treated human oral cancer cells, indicating that CCE induces protective autophagy and apoptosis [60]. Furthermore, 2’-hydroxcinnamaldehyde (HCA) also induces apoptosis and protective autophagy in human head and neck cancer cells [61]. However, we demonstrated that CA mediates autophagy and cell death by stimulating cleavage caspase-3; phosphorylating AMPKα, LC3B, Beclin-1, and ATG5; and down-regulating p62 and mTOR phosphorylation in GC cells.

ER stress triggers diverse cell death-related pathways, such as intracellular Ca²⁺, MEKK1, ER membrane re-organization, and programmed cell death type II (autophagic cell death), in various cancer types [62–65]. Prolonged and excessive stimuli by flavonoids causes ER stress to initiate the apoptosis and autophagic cell death pathway [66]. PERK−eIF2α−ATF4−CHOP and IRE1−JNK/XBP1−CHOP signaling pathways are the two useful signposts of ER stress, which is regulated by ROS and calcium production [67]. Therefore, ER stress has been suggested as a potential strategy for diseases therapy. Recently, an emerging study has reported the use of phytochemicals, such as phenolic acids, stilbenes, tannins, coumarins, and flavonoids targeting ER stress, which have an anti-cancer effect [68]. Morusin, a flavonoid isolated in the root bark of Morus australis (Moraceae), has been suggested to activate ER stress-mediated cell death by enhancing GRP78, IRE1α, and CHOP as well as phosphorylation of eIF2α and Ca²⁺ ion production in epithelial ovarian cancer cells [69]. Baicalein causes ER stress-induced cell death in hepatocellular
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Fig. 6 Targeting ER stress blocks cell death through the inhibition of autophagy in cinnamaldehyde-induced GC cells. a–d NCI-N87 and MKN-74 cells were transfected with PERK siRNA and treated with CA (50 μg/mL, 24 h). Cell viability, LDH release, and Ca\(^{2+}\) release were determined by WST-1, LDH, and Ca\(^{2+}\) assays, respectively; \(^*P<0.05\). p-PERK, PERK, p-eIF2α, eIF2α, and CHOP were detected using Western blotting analyses. β-actin was used as a protein loading control. e–h NCI-N87 and MKN-74 cells were transfected with control or CHOP siRNA in the presence or absence of CA (50 μg/mL, 24 h). Cell viability, LDH release, and Ca\(^{2+}\) release were determined by WST-1, LDH and Ca\(^{2+}\) assays, respectively; \(^*P<0.05\). The protein expression of CHOP and LC3B was detected using Western blotting analyses. β-actin was used as a protein loading control; \(^*P<0.05\).

carcinoma cells. It upregulated the expression of IRE1α, CHOP and GRP78 and the phosphorylation of PERK, JNK and eIF2α through calcium release, suggesting that it mediates ER stress [70]. HCA, the selective hydrogenation of CA, upregulated the glucose-regulated protein 78 (GRP78), GADD153, heme oxygenase-1 (HMOX1), homocysteine-responsive ER-resident ubiquitin-like domain member 1 protein (HERPUD1), Bax, and cytochrome c in several human cancer cells, indicating that HCA induces ER stress-mediated cell death via ROS release [71]. We found that CA has shown significant anti-cancer activity and triggers cell death via the PERK–CHOP axis in GC cells. CA exerts the phosphorylation of PERK and eIF2α and the accumulation of ATF4 and CHOP in a dose- and time-dependent manner. Thapsigargin (TG), an ER stress inducer to raise the intracellular calcium levels, mediates higher cell death in CA-treated GC cells compared to cells treated with TG or CA alone. In addition, targeting ER stress, including PERK- and CHOP-specific siRNAs, inhibited CA-induced cell death. Herein, our results indicated that CA increased intracellular Ca\(^{2+}\) release and cell death through ER stress.

Hot topics in increasing reports indicate a biological mechanistic interplay between the ER stress and autophagy [72]. Both LC3B and p62 interact with each other and are central factors of the autophagosome for the autophagy process [73]. Recent reports suggested that inducing ATF4 in the ER stress pathway frequently induces autophagic cell death by binding with a cyclic AMP response element site on the proximal promoter of autophagy-related genes, including LC3B, ATG5, and p62 [74]. Euchromatic histone–lysine N-methyltransferase (G9a) has been studied to serve functional roles in cell differentiation and tumor growth, and G9a was found to be upregulated under hypoxia response [75]. Furthermore, G9a binds to the promoters of autophagy-related genes, such as LC3B, Beclin-1, HIF-1, and WIP1, and its binding inhibits the autophagy process [76]. In the relationship among ER stress, autophagy, and G9a, we hypothesize that G9a may act as an activator by binding at Beclin-1 and LC3B promoter and that ATF4 binding may block G9a binding at LC3B promoter. HDAC inhibitor often exerts the downregulation of G9a via epigenetic modification, and both HDAC and G9a inhibitors mediate autophagic cell death [77]. BIX01294, a G9a inhibitor, induces autophagic cell death by dissociating G9a binding at LC3B promoter in colon cancer cells [78]. Our previous reports indicated that kaempferol, an inhibitor of HDAC and a flavonoid, mediates ER stress and autophagic cell death by inhibiting G9a binding at LC3B promoter in GC cells [79]. These findings suggested that CA induces ER stress and autophagic cell death by inhibiting G9a binding at LC3B promoter in GC cells.
CONCLUSION
In conclusion, our results identified that CA mediates ER stress and autophagic cell death via the PERK–CHOP signaling pathway, inhibition of G9a binding on Beclin-1 and LC3B promoter, and dissociation of Bcl-2–Beclin-1 in GC cells. A broader study of the biological mechanism of CA may suggest useful anti-cancer therapeutic strategies.

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AUTHOR CONTRIBUTIONS
TWK designed and performed most experiments. TWK also discussed the data and wrote the manuscript.

ADDITIONAL INFORMATION
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