Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Ginkgolide B Inhibits EMT and Promotes Pyroptosis in Gastric Cancer via AKT/mTOR Pathway

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Introduction: Gastric cancer (GC) remains a leading cause of cancer-related mortality worldwide, necessitating the exploration of novel therapeutic agents to improve patient outcomes. This study elucidates the anti-cancer properties of Ginkgolide B (GGB), a diterpenoid lactone derived from Ginkgo biloba, in both in vitro and in vivo models of GC.

Methods and Results: Using AGS and HGC-27 cell lines, we assessed GGB's impact on cellular proliferation, colony formation, migration, invasion, apoptosis, and pyroptosis. GGB exhibited significant dose- and time-dependent inhibition of cell proliferation and colony formation, with no cytotoxicity observed in normal gastric epithelial cells. Furthermore, GGB markedly suppressed migration and invasion, and induced apoptosis and pyroptosis, as evidenced by increased Bax and GSDMD expression and decreased Bcl-2 levels. In vivo, GGB treatment significantly reduced tumor growth in a nude mouse xenograft model and modulated EMT markers, decreasing PCNA and N-cadherin levels while increasing E-cadherin expression. Mechanistically, GGB's anti-cancer effects were mediated through the deactivation of the PI3K/AKT/mTOR signaling pathway.

Conclusion: These findings underscore the potential of GGB as a promising therapeutic agent for GC, warranting further clinical evaluation.

Keywords: gastric cancer, Ginkgolide B, EMT, pyroptosis, PI3K/AKT/mTOR

Introduction

Gastric cancer (GC) is one of the most prevalent and lethal cancers worldwide, ranking among the leading causes of cancer-related mortality.[1] Despite significant advancements in cancer therapies, survival rates for GC have seen minimal improvement over the past few decades.[2,3] The tumorigenesis of GC involves numerous genetic and epigenetic alterations that drive proliferation, invasion, and metastasis.[4,5] Thus, a deep understanding of the molecular mechanisms underlying gastric carcinogenesis and the identification of novel therapeutic agents are crucial for enhancing treatment outcomes.

In the realm of personalized and complementary medicine, bioactive phytochemicals have emerged as promising candidates for cancer treatment.[6–8] Ginkgolide B (GGB), a diterpenoid lactone derived from the leaves of Ginkgo biloba, has been traditionally used to treat respiratory illnesses, cardiovascular diseases, and neuropathies.[9–11] Recent studies have highlighted GGB's potential in combating various cancers, including lung cancer,[12] colon cancer[13] and oral cancer.[14] In the aforementioned literature, GGB may target the Wnt/β-catenin signaling pathway, inducing apoptosis in cancer cells and inhibiting metastasis. However, its antitumor effects against GC have not yet been explored.

Received: 6 September 2024
Accepted: 8 March 2025
Published: 2 April 2025

This study aims to investigate the anticancer effects of GGB on GC both in vitro and in vivo. Furthermore, we seek to elucidate the mechanisms by which GGB inhibits epithelial-mesenchymal transition (EMT) and promotes pyroptosis in GC cells.

Materials and Methods

Ethics Statement

All animal procedures were approved by the Committee of Animal Experimentation and the Ethics Committee of Peking University Cancer Hospital & Institute, Capital Medical University, and Beijing Shijitan Hospital (Approval No.KYD-2024-0002-002). The experiments adhered to NIH guidelines for animal care and use and complied with the Declaration of Helsinki.

Antibodies and Reagents

Ginkgolide B (GGB) and Dimethyl sulfoxide (DMSO), and corn oil were obtained from Sigma-Aldrich. The antibodies used were: anti-GSDMD, anti-Bax, anti-Bcl-2, anti-β-actin, anti-p-Akt, anti-Akt, anti-p-mTOR, anti-mTOR, anti-E-cadherin, and anti-N-cadherin (1:1000 dilution), and HRP-labelled goat anti-mouse IgGs (1:2000 dilution), all from Abcam.

Cell Lines and Culture

Normal human gastric epithelium cell line GES-1 and human gastric cancer cell lines AGS, HGC-27 were obtained from the Cell Bank of Peking Union Medical College. These cells were cultured in RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) in a humidified atmosphere with 5% CO2 at 37°C.

Cell Proliferation Assay (CCK-8)

Cell growth inhibition was measured using a CCK-8 kit (KeyGEN BioTECH) per the manufacturer's instructions. Cells in the logarithmic growth phase were seeded into 96-well plates at a density of 5×10^3 cells per 200 μL and incubated in a 37°C, 5% CO2 atmosphere. After 24 h, the medium was replaced with fresh medium containing various concentrations of GGB for 24, 48, or 72 h. Two hours before the end of each time point, 10 μL CCK-8 was added to each well and incubated for another 2 h. Optical density at 450 nm was recorded using an enzyme-linked immunosorbent assay plate reader (Bio-Rad) . The inhibition curves of GGB for each cell type were generated. Each experiment was repeated five times.

Colony Formation Assay

Cells were treated with vehicle control or specified concentrations of GGB for 48 h. After trypsinization, 200 cells per well were dispensed into 6-well plates and incubated for 14 days without medium change. Colonies were fixed with 10% formaldehyde for 10 minutes and stained with Giemsa solution for 15 minutes. Colonies with more than 50 cells were counted under a dissecting microscope. Each experiment was repeated five times.

Cell Migration Assay (Wound Healing Assay)

Cell migration was assessed using a wound healing assay. Cells at 90% confluency were incubated in 6-well plates with vehicle control or specified concentrations of GGB for 48 h. A sterile 200-μL pipette tip was used to create a scratch in the cell monolayer. Debris was washed away with PBS. Cells were treated with serum-free medium for 48 h. Images were captured at 0 h and 48 h using an inverted microscope, and wound healing was quantified using Image J software (Scion). Each experiment was repeated five times.

Cell Invasion Assay (Transwell Assay)

Cell invasion was evaluated using 24-well Biocoat cell culture inserts (BD Biosciences) with 8-μm pore membranes coated with Matrigel (1 mg/mL; BD Biosciences). Membranes were coated and incubated for 6 h at 37°C. Cells treated with vehicle control or specified concentrations of GGB for 48 h were seeded in the upper chamber with serum-free

medium, and medium with 10% FBS was added to the lower chamber. After 24 h, cells were washed with PBS, fixed with paraformaldehyde, and stained with Giemsa. Cells on the upper surface were removed, and invading cells on the lower surface were photographed. Each experiment was repeated five times.

Apoptosis and Pyroptosis Analysis by TUNEL Assay

Cell apoptosis and pyroptosis was detected by TUNEL assay. AGC cells were cultured in 12-well plates for 24 h followed by the exposure of indicated concentrations of GGB for 48 h. Then cells were harvested, apoptosis and pyroptosis was measured using TUNEL cell in situ detection kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions. Positively stained cells were counted using a microscope. Each experiment was repeated five times.

Western Blot Analysis

Protein lysates were prepared on ice using ice-cold RIPA buffer (Beyotime) with protease inhibitor cocktail (Roche). Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad). Samples were denatured at 100°C for 10 minutes, separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were blocked with 5% fetal bovine serum for 2 h at room temperature, then incubated with primary antibodies at 4°C overnight. After washing with TBST, membranes were incubated with HRP-labelled goat anti-mouse IgG (1:2000; Abcam) at 37°C for 1 h. Chemiluminescent signals were detected using Clarity™ ECL Western Substrate (Bio-Rad) and quantified with Quantity One Software Version 4.1.1 (Bio-Rad). GAPDH served as the loading control. Each experiment was repeated five times.

Animal Study

Male Balb/c nude mice (20–22 g, 6–8 weeks old) were obtained from the experimental animal ministry of Capital Medical University and acclimated for one week. Mice (n=18) were injected subcutaneously with 1×10^6 AGS cells in 100 μL of Matrigel and PBS mixture (1:1) into the right axillary fossa. Mice were randomized into three groups: vehicle control (PBS), low-dose GGB (30 mg/kg/day), and high-dose GGB (60 mg/kg/day). GGB was dissolved in PBS and administered intragastrically. When tumors reached approximately 100 mm^3, treatment began. Tumor sizes were measured every 3 days, and volumes calculated using the formula L×S^2×0.5, where L is the longest diameter and S is the shortest diameter. After 28 days, mice were anesthetized, sacrificed, and tumors were weighed. Samples were collected for further analysis.

Immunofluorescence Staining

Xenograft tumors were fixed in 10% phosphate buffered formalin and paraffin-embedded for immunofluorescence staining. Sections (5 μm) were deparaffinized, rehydrated, and treated with 3% H2O2. Sections were incubated with primary antibodies against PCNA or E-cadherin (1:100, Abcam) overnight at 4°C, followed by secondary antibodies (Invitrogen). Sections were mounted with DAPI and analyzed using a fluorescence microscope. Each experiment was repeated five times.

Statistical Analysis

Each assay was independently repeated five times, each repeat was performed as a separate, independent experiment. Statistical analyses were performed using SPSS (22.0), with data presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for group comparisons. A p-value < 0.05 was considered statistically significant and was denoted by * or #, a p-value < 0.01 was considered highly significant and was denoted by ** or ##, and a p-value < 0.001 was considered extremely significant and was denoted by *** or ###.

Results

Ginkgolide B Suppresses Proliferation in Gastric Cancer Cells in a Dose- and Time-Dependent Manner

To investigate the effects of GGB on the biological functions of gastric cancer cells, we conducted cell proliferation assays (CCK-8) and colony formation assays. The CCK-8 assay revealed that GGB inhibited the proliferation of AGS

and HGC-27 cells in a dose- and time-dependent manner (Figure 1B-E), with cell viability decreasing as GGB concentration increased (0 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM, 200 μM) and exposure time extended (24h, 48h, 72h). Importantly, GGB did not exhibit cytotoxicity towards normal gastric epithelium GES-1 cells (Figure 1A). Colony formation assays further confirmed that GGB treatment reduced the number of surviving GC cells, demonstrating a dose-dependent inhibition of cell colony formation (Figure 2A-D).

Ginkgolide B Inhibits Migration and Invasion of Gastric Cancer Cells in a Dose-Dependent Manner

To assess the impact of GGB on cell migration, we employed a wound healing assay. The results showed that GGB treatment (0, 20, and 50 μM for 24 h) significantly inhibited cell migration in a dose-dependent manner (Figure 3A-D). Similarly, the transwell assay revealed that GGB treatment reduced the invasion capability of GC cells, consistent with the migration assay results (Figure 4A-D). Collectively, these findings indicate that GGB inhibits migration and invasion of GC cells in a dose-dependent manner.

Ginkgolide B Promotes Pyroptosis and Apoptosis in Gastric Cancer Cells in a Dose-Dependent Manner

Pyroptosis and apoptosis were evaluated using TUNEL assays. The results indicated that GGB treatment significantly induced apoptosis and pyroptosis in GC cells compared to the vehicle control group (Figure 5A-D). The rates of apoptosis and pyroptosis increased in a dose-dependent manner with GGB treatment. Western blot analysis confirmed these findings, showing increased expression of apoptotic proteins (Bax) and pyroptosis proteins (pro-GSDMD, N-GSDMD), and decreased expression of anti-apoptotic protein (Bcl-2) (Figure 6A-C). These results suggest that GGB effectively induces apoptosis and pyroptosis in GC cells in a dose-dependent manner.

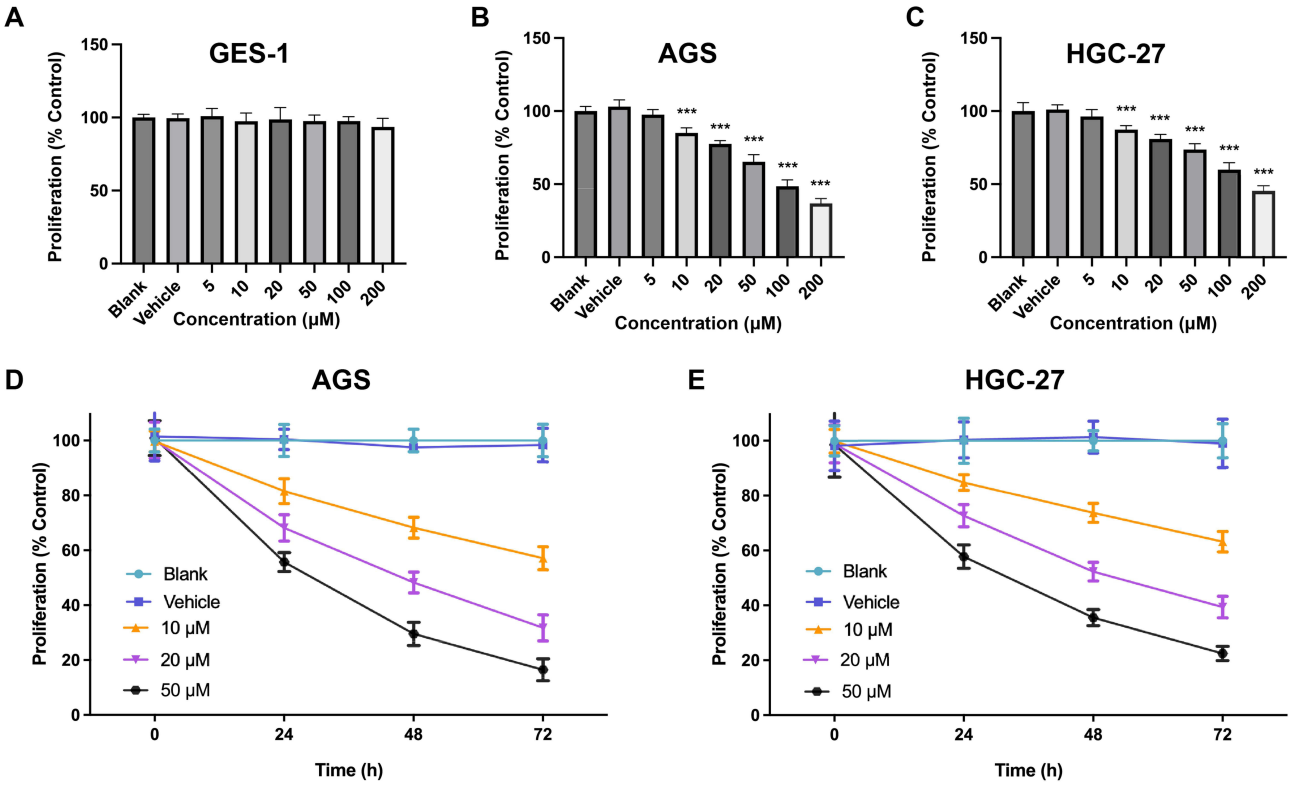

Figure 1 GGB suppresses proliferation in gastric cancer cells in a dose- and time-dependent manner. (**A–C**) CCK-8 assay: indicated cells were treated with different concentrations of GGB as indicated. Cell viability was determined in 24h. (**D**), (**E**) CCK-8 assay: indicated cells were treated with different concentrations of GGB as indicated. Cell viability was determined in 24, 48, 72h. ***P<0.001, compared with blank control group.

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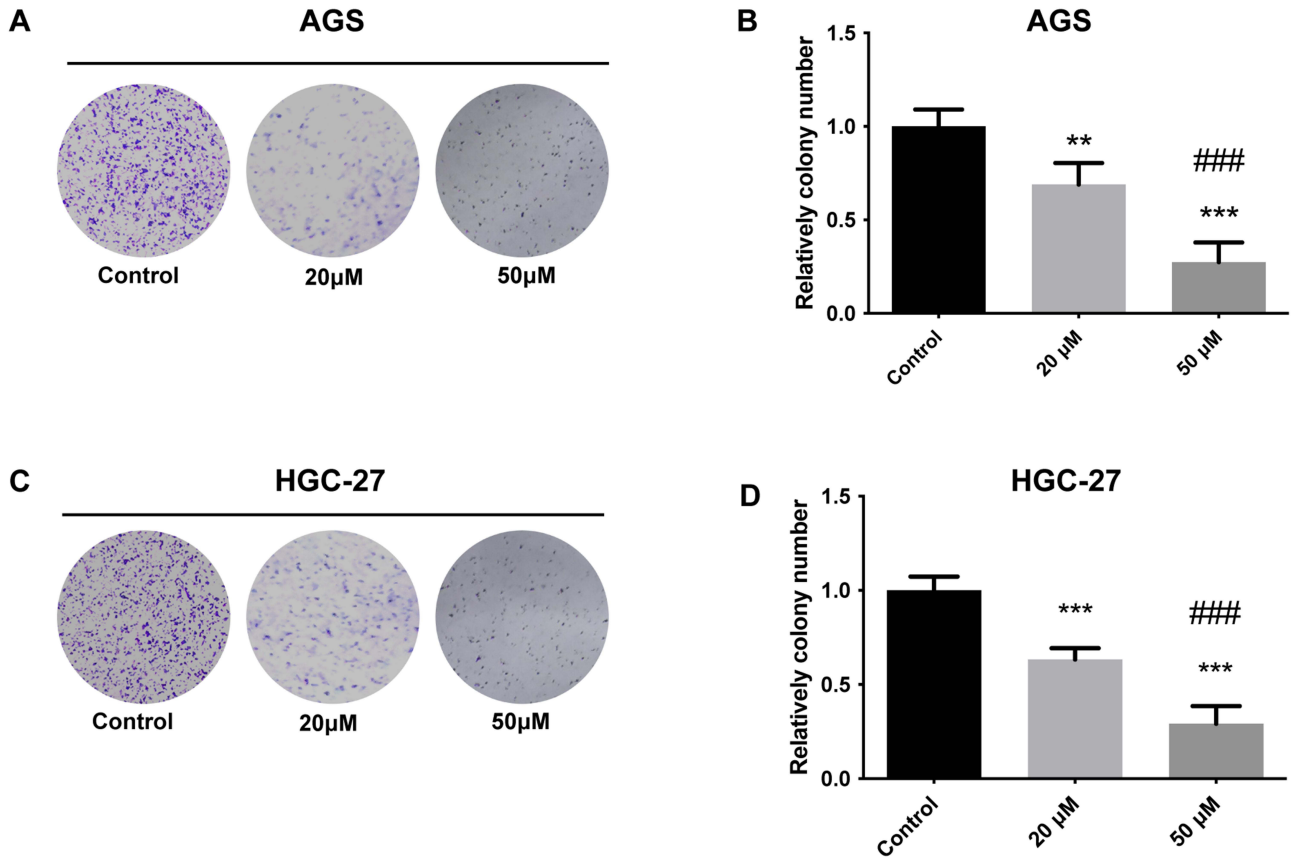

Figure 2 GGB inhibits colony formation in gastric cancer cells in a dose-dependent manner. (**A-D**) colony formation assay was utilized to evaluate the capability of indicated cells with indicated concentrations of GGB treatment on colony formation ability. **P<0.01, ***P<0.001, compared with vehicle control group. ###P<0.001, compared with 20 μM group.

GGB Inhibits Gastric Cancer via Suppressing EMT and the PI3K/AKT/m-TOR Signaling Pathway

To explore the mechanism of GGB's action on GC, we examined the expression of EMT markers (E-cadherin, N-cadherin) and components of the PI3K/AKT/m-TOR signaling pathway by Western blotting (Figure 7A). The results demonstrated that GGB treatment significantly decreased the expression ratios of p-AKT/AKT and p-m-TOR/m-TOR, while increasing the expression ratio of E-cadherin/N-cadherin in a dose-dependent manner (Figure 7B-C). These findings indicate that GGB may suppress EMT and promote apoptosis and pyroptosis in GC cells by deactivating the PI3K/AKT/m-TOR signaling pathway.

GGB Inhibits Tumor Growth and EMT in Nude Mice Xenograft Models of GC

To evaluate the in vivo effects of GGB on GC, AGS cells were subcutaneously injected into nude mice, followed by daily treatment with vehicle control (PBS) or GGB at indicated doses (30 mg/kg/day and 60 mg/kg/day). Tumor sizes were measured every three days (Figure 8B), and after 28 days, the mice were sacrificed, and tumors were collected and weighed (Figure 8A). GGB treatment significantly slowed tumor growth compared to the vehicle control, with higher doses of GGB (60 mg/kg/day) leading to greater reductions in tumor size (Figure 8C). Notably, GGB at doses up to 60 mg/kg was well tolerated, with no observed side effects such as poor mental state or weight loss (Figure 8D). Immunofluorescence staining revealed that GGB treatment downregulated PCNA (a proliferation marker) and upregulated E-cadherin (an EMT marker) in a dose-dependent manner (Figure 8E-H). These results suggest that GGB effectively inhibits tumor growth and EMT in GC in vivo.

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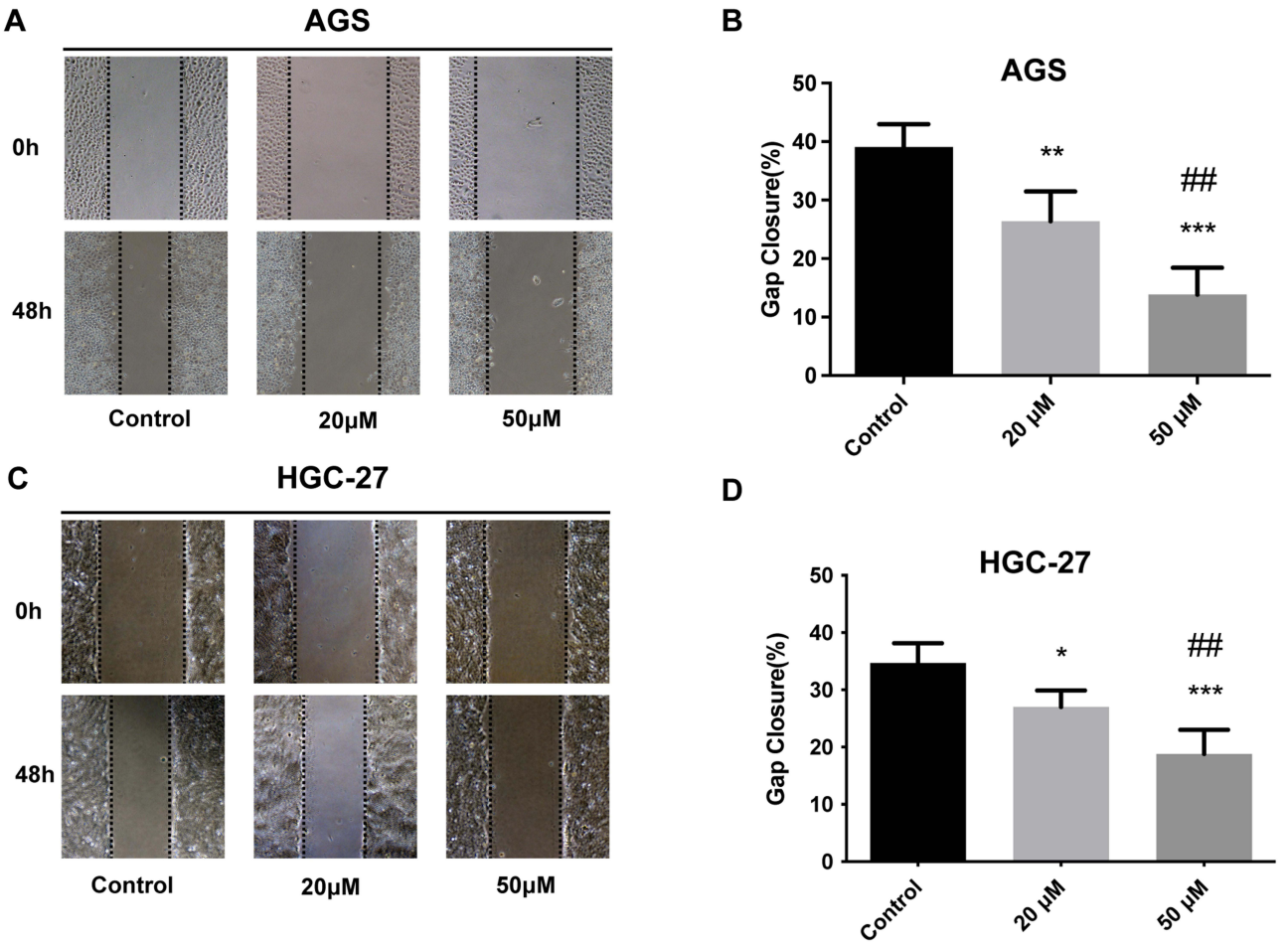

Figure 3 GGB inhibits migration of AGS and HGC-27 cells. (**A-D**): wound healing assay were carried out to measure the migratory ability of indicated cells. *P<0.05, **P<0.01, ***P<0.001, compared with vehicle control group. ##P<0.01, compared with 20 μM group.

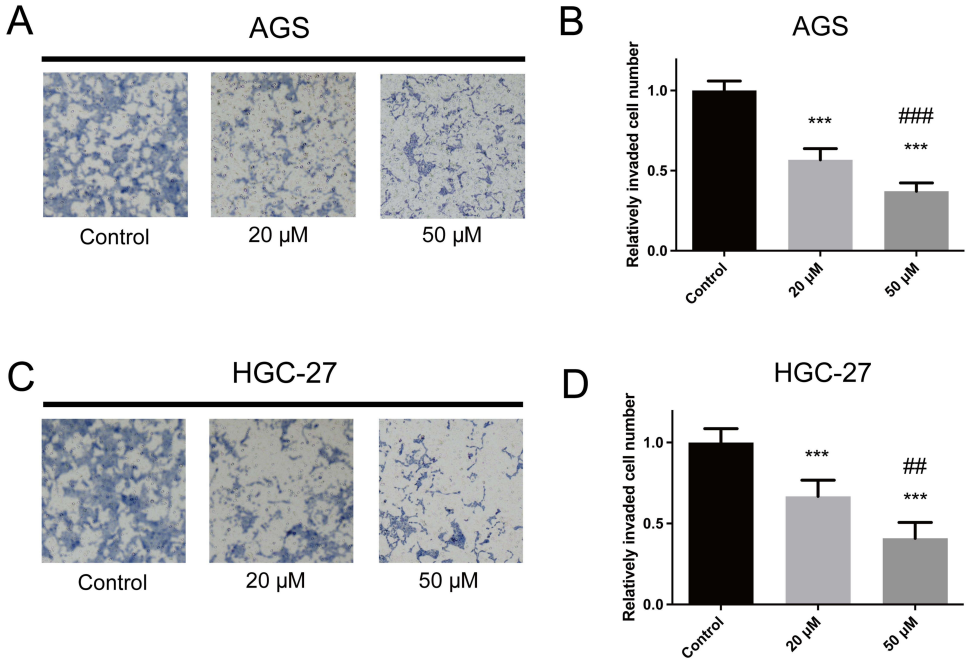

Figure 4 GGB inhibits invasion of AGS and HGC-27 cells in a dose-dependent manner. (**A-D**) transwell assays were carried out to measure the invasive ability of indicated cells. ***P<0.001, compared with vehicle control group. ##P<0.01, ###P<0.001, compared with 20 μM group.

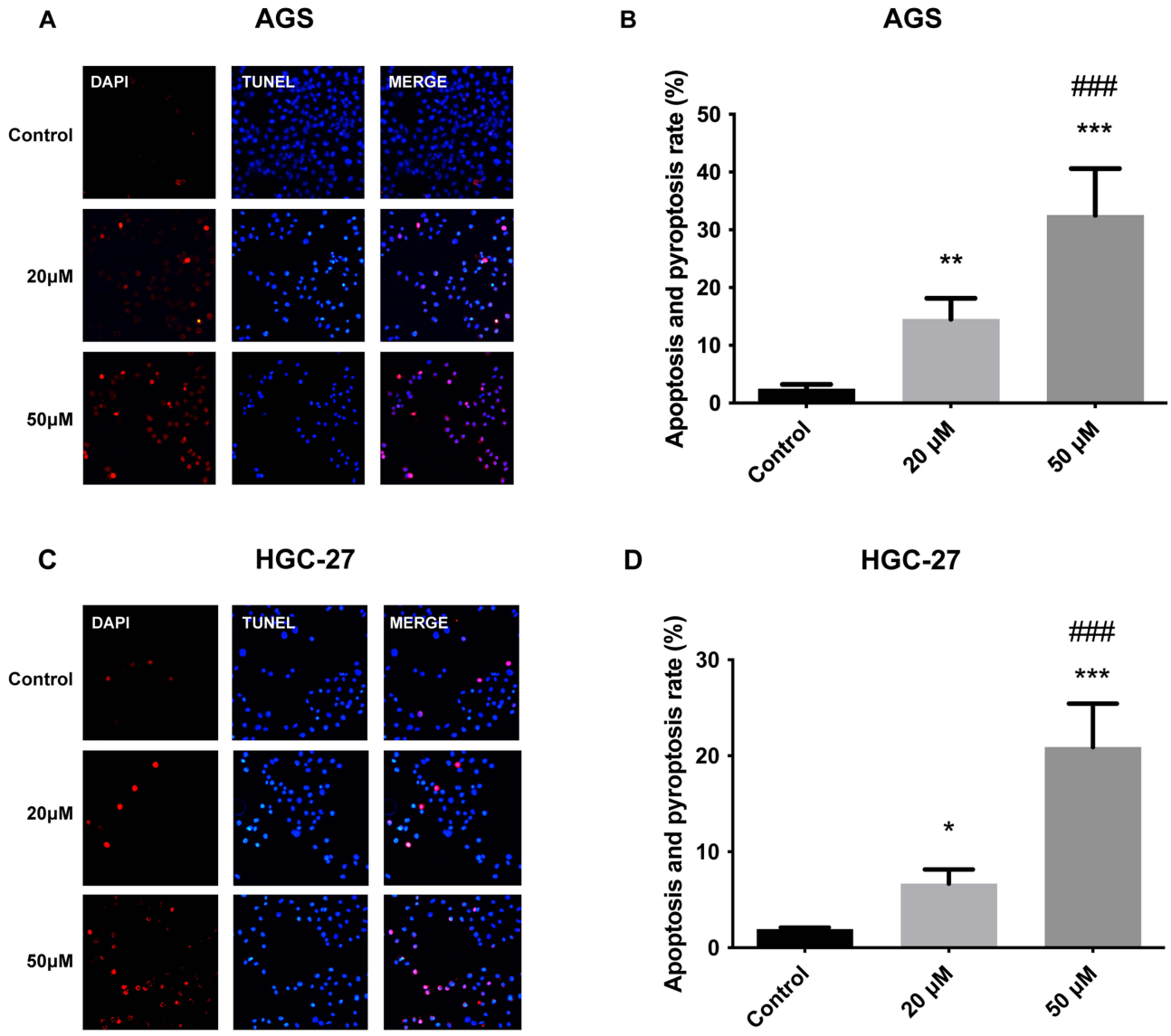

Figure 5 GGB promotes apoptosis and pyroptosis of AGS cells in a dose-dependent manner. (**A-D**) TUNEL assay was used to assess the apoptosis and pyroptosis rate of indicated cells under indicated concentration of GGB treatment. *P<0.05, **P<0.01, ***P<0.001, compared with vehicle control group. ###P<0.001, compared with 20 μM group.

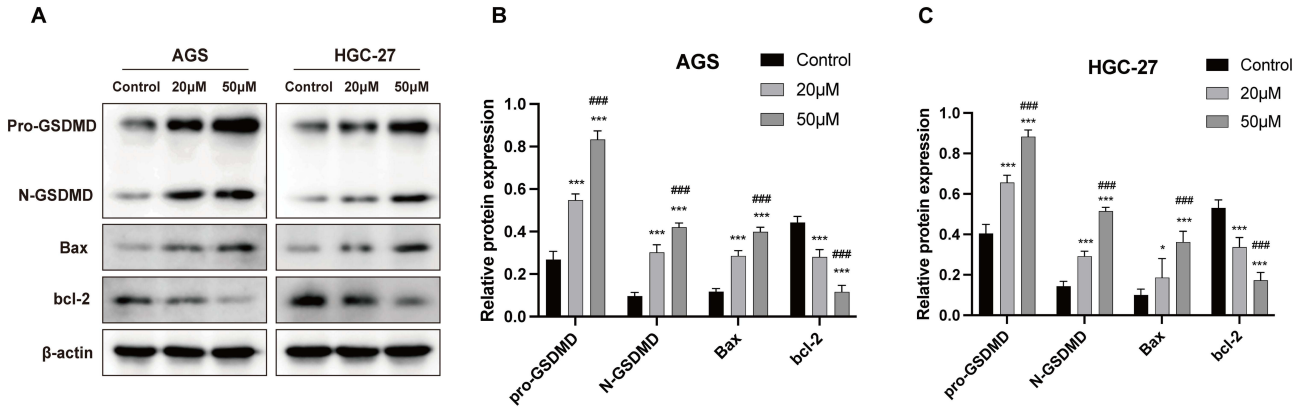

Figure 6 Modulations on the expression of proteins involved in pyroptosis and apoptosis. (**A-C**) associated proteins that participated in the pyroptosis and apoptosis were examined by the Western blot analysis. *P<0.05, ***P<0.001, compared with vehicle control group. ###P<0.001, compared with 20 μM group.

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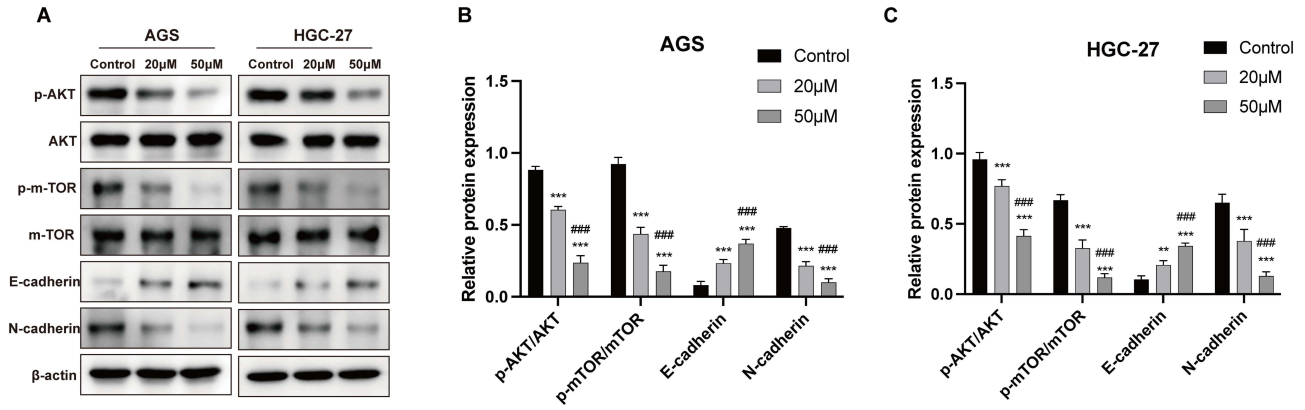

Figure 7 Modulations on the expression and phosphorylation of proteins involved in PI3K/AKT/m-TOR signal pathway and EMT process. (**A-C**) The Western blot assay was applied to examine the expression levels of corresponding proteins in the EMT process and PI3K/ AKT/ m-TOR signaling pathway. **P<0.01, ***P<0.001, compared with vehicle control group. ###P<0.001, compared with 20 μM group.

Discussion

Gastric cancer (GC) remains a prevalent and lethal malignancy worldwide, presenting significant challenges despite advances in management.[2,15] Current therapeutic strategies often yield suboptimal outcomes, particularly in advanced stages.[3,16] However, the present comprehensive therapeutic measures cannot achieve satisfactory results. Consequently, the exploration of naturally-occurring compounds as alternative treatments has garnered considerable interest.

Epidemiological evidence suggests that diets rich in vegetables, fruits, olive oil, grains, and fish, characteristic of the Mediterranean diet, may reduce cancer incidence.[17–21]

Ginkgolide B (GGB) is a diterpenoid lactone extracted from the leaves of Ginkgo biloba, a traditional medicinal plant. Ginkgolides, particularly ginkgolide B, are renowned for their potent pharmacological activities, including neuroprotective, anti-inflammatory, and antioxidative properties.[22–25] GGB functions primarily as a platelet-activating factor (PAF) receptor antagonist, thereby mitigating inflammatory responses and reducing platelet aggregation.[26,27]

Recent research has highlighted GGB's potential antitumor effects, including the induction of apoptosis, cell cycle arrest, and angiogenesis inhibition. By modulating critical signaling pathways such as PI3K/Akt, MAPK, and NF-κB.[28–30] Additionally, ginkgolide B has demonstrated the ability to enhance the efficacy of conventional chemotherapeutic agents, suggesting its potential as an adjunct therapy in cancer treatment.[31,32] However, its antitumor activity against GC had not been previously investigated.

Our study demonstrates that GGB significantly inhibits the growth of AGS and HGC-27 gastric cancer cell lines in vitro. This effect correlates with increased apoptosis and pyroptosis, and reduced proliferation, colony formation, migration, and invasion in a dose-dependent manner. Furthermore, GGB exhibited significant antitumor activity in vivo, reducing xenograft tumor growth in nude mice without evident toxicity, suggesting good tolerability.

The PI3K/AKT/m-TOR signaling pathway is integral to tumorigenesis and progression, promoting cell survival and epithelial-mesenchymal transition (EMT).[33–36] Aberrant activation of this pathway is linked to increased cell proliferation and survival, mediated by oncogenes and growth factors such as VEGF and c-Myc.[37,38] Inhibition of this pathway by therapeutic agents can induce pyroptosis, contributing to their antitumor effects.[39–42] Our results indicated that GGB deactivated the PI3K/AKT/m-TOR pathway in a dose-dependent manner, thereby inhibiting proliferation and promoting apoptosis and pyroptosis.

EMT is crucial in cancer metastasis, characterized by a switch from E-cadherin to N-cadherin expression.[43–45] Our findings show that GGB treatment increases E-cadherin and decreases N-cadherin expression in a dose-dependent manner. This modulation of EMT markers, along with the regulation of apoptosis and pyroptosis-related proteins, underscores GGB's therapeutic potential against GC (Figure 7A-C).

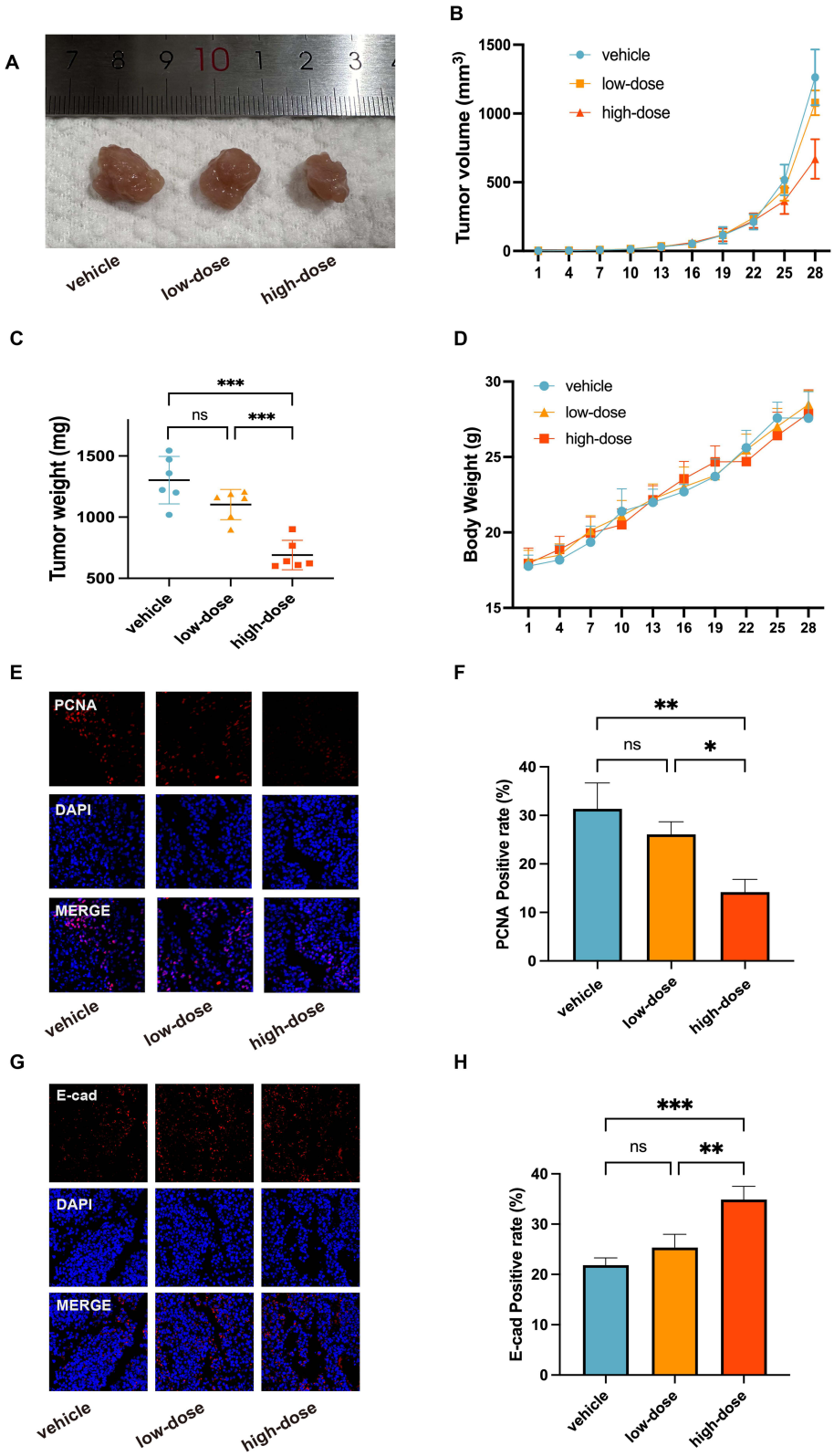

Figure 8 The anti-cancer effect of GGB in nude mice xenograft models of AGS. In the study, the low-dose group received GGB at a dosage of 30 mg/kg/day, while the high-dose group received GGB at 60 mg/kg/day. (**A**) Photographs of dissected tumors in nude mice. (**B**) the tumor volume fluctuation of the mice was measured every three days. (**C**) the tumor weight of the mice was measured in the end of experiment. (**D**), the body weight fluctuation of the mice was measured every three days. (**E** and **F**) immunofluorescence staining detected PCNA positive cells in xenograft tumor tissue. (**G** and **H**), immunofluorescence staining detected E-cadherin-positive cells in xenograft tumor tissue. *P<0.05, **P<0.01, ***P<0.001, compared with indicated group.

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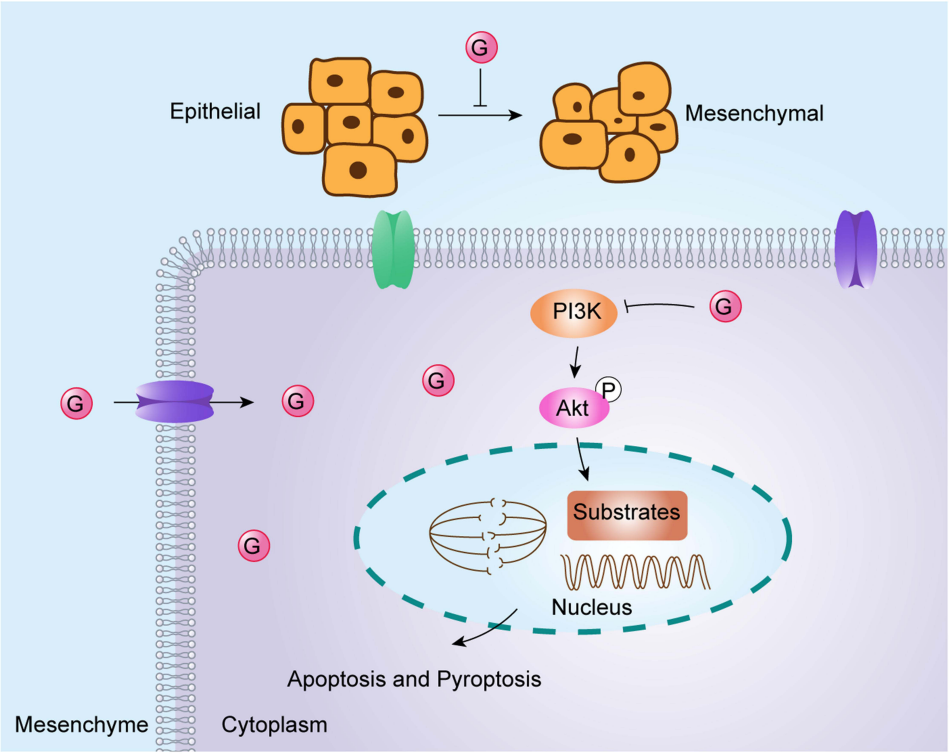

Scheme 1 Schematic diagram illustrating the role of GGB in modulating the PI3K/AKT/mTOR signaling pathway, the epithelial mesenchymal transition (EMT) process, and pyroptosis. As shown: (1) GGB inhibits the PI3K/Akt signaling pathway; (2) GGB reverses the EMT process; (3) GGB induces apoptosis and pyroptosis. "G" is an abbreviation for GGB; The "→" symbol indicates activation or induction, and the "⊥" symbol indicates inhibition or blockade.

In summary, our study reveals that GGB exerts a multifaceted anticancer effect by targeting EMT, apoptosis, pyroptosis, and the PI3K/AKT/m-TOR pathway. These findings provide a basis for considering GGB as a novel therapeutic option for GC, warranting further clinical investigation.

Conclusions

In summary, our study is the first to demonstrate that Ginkgolide B (GGB) exhibits significant anticancer effects on gastric cancer (GC) both in vitro and in vivo. Our results indicate that GGB suppresses epithelial-mesenchymal transition (EMT) and promotes apoptosis in GC cells through the deactivation of the PI3K/AKT/m-TOR signaling pathway. As depicted previously, a comprehensive molecular mechanism underlying the inhibitory effect of Ginkgolide B on EMT and promotion of pyroptosis in gastric cancer via the AKT/mTOR pathway is illustrated (Scheme 1). These findings suggest that GGB could be a promising therapeutic agent for gastric cancer. However, further clinical trials are necessary to validate the efficacy and safety of GGB in the treatment of gastric cancer. In order to fully realize its clinical value, future research might focus on exploring combination therapies with other agents and evaluating its efficacy in clinical practice.

Abbreviations

GC, Gastric cancer; GGB, Ginkgolide B; EMT, Epithelial mesenchymal transition; PI3K, Phosphatidylinositol 3 kinase; mTOR, Mammalian target of rapamycin; CCK-8, Cell Counting Kit – 8; PCNA, Proliferating Cell Nuclear Antigen.

Funding

This research was financially supported by Peking University Cancer Hospital Construction Project (2024) for Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education).

Disclosure

The authors report no conflicts of interest in this work.

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