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# Ganoderic acid a potentiates cisplatin's cytotoxicity on gallbladder cancer cells by promoting DNA damage and inhibiting cell stemness

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## Abstract

**Background** Ganoderma acid A (GAA), a triterpenoid compound from *Ganoderma lucidum*, has gained attention for its anti-tumor properties. Herein, we hypothesized that GAA may enhance cisplatin's (DDP) anticancer effect in gallbladder cancer (GBC) cells by promoting DNA damage response, particularly through upregulation of DNA damage markers such as γH2AX, p-ATM, p-ATR, and p-p53, and reducing cell stemness by downregulating stemness markers like SOX2, Oct4, and NANOG.

**Materials and methods** The human GBC cell line GBC-SD and human gallbladder epithelial cell line HGBEC were cultured in RPMI-1640 and DMEM/F12 media with 10% fetal bovine serum. Cells were treated with 2 μM DDP and 60 μM GAA for 24 h. To evaluate the toxicity of GAA in normal cells, HGBEC cells were treated under the same conditions. Cell viability was assessed by CCK-8 assay, and colony formation was measured in 6-well plates. Apoptosis was evaluated by TUNEL assay, and DNA damage was assessed using comet assay. Stemness was analyzed by spheroid formation and CD44 immunofluorescence staining. Western blot analysis was performed to evaluate the expression of apoptotic, stemness, and DNA damage markers (Bax/Bcl-2, cleaved-caspase 3, SOX2, Oct4, NANOG, γH2AX, p-ATM, p-ATR, p-p53).

**Results** The results showed that GAA significantly reduced GBC-SD cell viability in a concentration-dependent manner ($p < 0.05$). The combined treatment of GAA and DDP further decreased cell viability, with the DDP IC50 value reduced from 8.98 μM to 4.07 μM ($p < 0.05$). Colony formation was significantly inhibited ($p < 0.05$), and apoptosis increased, as assessed by TUNEL assay ($p < 0.05$). Western blot analysis revealed increased pro-apoptotic proteins Bax/Bcl-2 and cleaved-caspase 3($p < 0.05$). The expression of stemness markers SOX2, Oct4, NANOG, and DNA damage markers γH2AX, p-ATM, p-ATR, and p-p53 was significantly altered ($p < 0.05$). Specifically, p53 expression was significantly increased, indicating enhanced DNA damage response ($p < 0.05$).

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**Conclusion** GAA can significantly enhance the anticancer effects of DDP on GBC cells by inhibiting DNA damage response and cell stemness, supporting GAA as an adjuvant treatment for GBC and warrants further validatory preclinical studies.

**Keywords** Ganoderic acid A, Cisplatin, Gallbladder cancer, DNA damage response, Cell stemness, Apoptosis, Anticancer effect

## Introduction

Gallbladder cancer (GBC) is an aggressive malignancy with particularly high incidence rates in regions such as South America, East Asia, and India [1, 2]. According to the Global Cancer Report, the annual incidence of GBC is approximately 1–2 cases per 100,000 people, with a higher rate observed in women compared to men [3]. Despite its overall low global incidence, GBC rates are significantly elevated in areas with a high prevalence of gallstones [4]. The early symptoms of GBC are often subtle, and most patients are diagnosed at advanced stages, resulting in poor treatment outcomes and a 5-year survival rate of less than 20% [5, 6]. This combination of high lethality and propensity for malignant metastasis underscores the urgent need for effective therapeutic strategies [7, 8].

Standard treatments for GBC include surgical resection, radiotherapy, and chemotherapy [9, 10], but due to the challenges in early detection, surgery is typically only feasible for patients in the early stages of the disease. For advanced-stage patients, chemotherapy, particularly with cisplatin (DDP), remains the primary treatment option [11, 12]. However, the effectiveness of DDP is often compromised by drug resistance, limiting its clinical benefit [13, 14]. Research shows that approximately 50–60% of GBC patients exhibit resistance to DDP, leading to poor treatment outcomes and low survival rates [15, 16]. This resistance is often mediated by multiple biological mechanisms, such as adaptive changes in cancer cells and activation of specific molecular pathways, which collectively drive tumor growth and chemotherapy evasion.

To overcome DDP resistance, Ganoderic acid A (GAA), a triterpenoid compound derived from the traditional Chinese medicine *Ganoderma lucidum*, has garnered significant attention in recent years [17, 18]. GAA exhibits a wide range of pharmacological activities, including anti-inflammatory, antioxidant, and antiproliferative effects [19, 20]. In various cancer types, GAA has been shown to inhibit cancer cell proliferation and induce apoptosis through multiple pathways, while exhibiting low toxicity to normal cells [21, 22]. Studies have reported that, GAA has shown potential to reduce cancer cell migration and invasion by activating apoptotic pathways [23, 24]. At the same time, GAA has been found to suppress the DNA damage response, which may play a crucial role in mitigating cancer cell resistance to chemotherapy drugs [25, 26].

However, the specific mechanisms by which GAA exerts its anticancer effects in GBC, particularly how it modulates cancer stemness and the DNA damage response to enhance DDP efficacy, remain unclear. Although studies have shown that GAA may improve chemotherapy outcomes through these mechanisms, further exploration is needed, especially regarding how GAA specifically acts in GBC cells.

Therefore, this study aims to investigate, using the GBC-SD cell line in vitro, the effects of GAA, both alone and in combination with DDP, on cell proliferation, apoptosis, stemness, and DNA damage, to further clarify the potential of GAA in overcoming DDP resistance.

## Materials and methods

### Cell culture and grouping

Human gallbladder epithelial cells (HGBEC) were obtained from the American Type Culture Collection (ATCC, CRL-3245) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a 5% $CO_2$ incubator. For experimental treatments, HGBEC cells were cultured in Roswell Park Memorial Institute (RPMI 1640) medium with 10% FBS. The Control group was treated with 0 μM GAA without any additional treatment, while the GAA group was treated with different concentrations of GAA (including 5 μM, 20 μM, 40 μM, 60 μM, and 80 μM) for the GAA group for 24 h.

The human GBC cell line GBC-SD was purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (SIBCB, CAS, SCSP-526). GBC-SD cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified incubator at 37 °C with 5% $CO_2$. For experimental treatments, GBC-SD cells were divided into four groups, as outlined below: [1] Control group: the cells were cultured in RPMI 1640 medium with 10% FBS without any additional treatment; [2] DDP group: GBC-SD cells were treated with 2 μM DDP for 24 h [27]; [3] GAA group: the cells were treated with 60 μM GAA [28–30] for 24 h, and [4] DDP + GAA group: GBC-SD cells were co-treated with 2 μM DDP and 60 μM GAA for 24 h.

HGBEC cells were cultured in DMEM for standard growth and in RPMI 1640 for experimental treatments to support drug handling. Similarly, RPMI 1640 was used

for GBC-SD cells to maintain optimal conditions during drug treatments. For cell line authentication, STR (Short Tandem Repeat) profiling was performed to verify the identity of both HGBEC and GBC-SD cell lines. Additionally, mycoplasma contamination was tested and found negative using a PCR-based mycoplasma detection kit. These steps were conducted to ensure the accuracy and reliability of the experimental results. The treatments were carried out in triplicate, and the cells were harvested at the designated time points for further analysis. All culture media and reagents were sourced from reputable suppliers, and cell viability and other endpoints were assessed as described in the following sections.

#### CCK-8 assay

Cells were seeded in 96-well plates at a density of 5000 cells/well and cultured in an incubator at 37 °C and 5% $CO_2$ for 24 h to ensure that the cells adhered to the wall before treatment. According to the experimental design, different concentrations of treatment groups were added and treated for 24 h. After drug treatment, 10 μL of CCK-8 solution (Beyotime, C0041) was added to each well and incubated for 1–2 h. After incubation, the absorbance (OD value) was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad, 168–1000). The cell viability was calculated by normalizing the absorbance of each treated group to the absorbance of the Control group (0 μM GAA group or untreated cells group), with viability (%) = (OD of treated cells / OD of Control cells) × 100%.

#### Cell colony formation assay

Cells were seeded in 6-well plates at a density of 500 cells per well and cultured in an incubator at 37 °C and 5% $CO_2$. According to the experimental design, different treatment groups were added after the cells adhered to the wall and cultured for 14 days. The culture medium was changed every 3 days during this period. After the culture, the cells were gently washed twice with PBS buffer (ThermoFisher Scientific, AM9624), fixed with 4% paraformaldehyde (Beyotime, P0099) for 15 min, and then washed again with PBS. After fixation, the cells were stained with 0.1% crystal violet solution (Beyotime, C0121) for 20 min, washed with PBS to remove excess staining solution, and finally dried in air. The formation of cell colonies was observed under a microscope. Colonies with a diameter greater than 0.5 mm were counted as one colony. The colony formation rate was quantified by counting the number of colonies in each well manually, and the colony formation rate was calculated as follows: Colony formation rate (%) = (number of colonies in treated group / number of colonies in untreated cells group) × 100%.

#### TUNEL

Cell apoptosis detection was performed using the TUNEL kit ( Guangzhou Yujia Biotechnology Co., Ltd, C1086). First, the treated cells were fixed in 4% paraformaldehyde solution (Beyotime, P0099) at room temperature for 30 min. After fixation, the cells were washed 3 times with PBS ( ThermoFisher Scientific, AM9624), 5 min each time. Then, the cells were treated with 0.3% Triton X-100 permeabilization solution (Beyotime, P0096-100 ml) for 5 min to increase the permeability of the cell membrane, followed by 3 washes with PBS. According to the instructions of the TUNEL kit, an appropriate amount of TUNEL reaction solution was added and incubated at 37 °C for 1 h, with the process protected from light. After incubation, the cells were washed 3 times with PBS, 5 min each time. Fluorescence images of TUNEL-positive cells were observed and photographed using a fluorescence microscope. The apoptotic cells were quantified by counting the number of TUNEL-positive cells and calculating the apoptosis rate as follows: Apoptosis rate (%) = (number of TUNEL-positive cells / total number of cells) × 100%.

#### Cell spheroidization experiment

Cells from each treatment group were collected 24 h after treatment. The cells were digested with trypsin (Pricella, PB180225), prepared into a single-cell suspension, and seeded in ultra-low attachment 6-well plates at a density of 1,000 cells per well. Spheroidization was induced using a serum-free medium composed of DMEM/F12, 20 ng/mL epidermal growth factor (EGF), 10 ng/mL basic fibroblast growth factor (bFGF), and B27 supplement. No antibiotics were added to the medium. Cells were cultured at 37 °C with 5% $CO_2$, and the culture medium was replaced every 3 days. After 10–14 days of culture, spheroid formation was assessed using an inverted microscope. The number of spheroids formed in randomly selected fields of view was counted, and only those with a diameter greater than 50 μm were considered. Each experimental group was repeated three times, and the average number of spheroids per group was recorded.

#### Immunofluorescence detection

Cells from the different treatment groups were collected after 24 h of treatment and fixed with 4% paraformaldehyde for 15 min at room temperature. Following fixation, the cells were gently washed twice with PBS and permeabilized with 0.1% Triton X-100 solution (Beyotime, P0096) at room temperature for 10 min. After permeabilization, the cells were blocked with 5% bovine serum albumin (BSA) for 30 min. The cells were then incubated overnight at 4 °C with a primary antibody against CD44 (1:100 dilution, Abcam, UK, ab254530). The following day, cells were washed three times with PBS for 5 min

each, and a fluorescently labeled secondary antibody (1:200 dilution) was applied and incubated at room temperature for 1 h in the dark. DAPI (4',6-diamidino-2-phenylindole) stain was added to the cells, and incubation was carried out at room temperature for 10 min. After washing three times with PBS, the cells were observed under a fluorescence microscope. The fluorescence intensity of CD44 was recorded, and the average fluorescence intensity in each treatment group was quantified using ImageJ.

### Comet assay

For the comet assay, treated cells were gently collected and prepared into a single-cell suspension. A 100 μL aliquot of the suspension was mixed with 0.5% low-melting point agarose and quickly dropped onto a glass slide pre-coated with 1% normal agarose. A coverslip was placed on top, and the slide was kept at 4 °C for 10 min to allow solidification. After solidification, the coverslip was removed, and the slide was placed in lysis buffer and incubated at 4 °C in the dark for 2 h. Following lysis, the slide was immersed in alkaline electrophoresis buffer for 20 min, followed by electrophoresis (25 V, 300 mA) for 20 min. After electrophoresis, the slide was neutralized with a neutralizing buffer and washed twice with distilled water. The slide was then stained with 1X SYBR Green (Beyotime, S7585) for 10 min at room temperature in the dark. Comet images were captured using a fluorescence microscope. DNA damage was quantified by measuring the tail DNA content (comet tail DNA level) and tail length (comet tail distance) of individual comets. ImageJ software or similar image analysis tools were used to calculate the average tail DNA content and tail distance for each treatment group, providing an assessment of the extent of DNA damage.

### Western blot

Western Blot was used to detect protein expression levels. Total cell protein was extracted with RIPA lysis buffer, and protein concentration was determined by the bicinchoninic acid assay (BCA) method. 30 μg protein per well was separated by SDS-PAGE gel electrophoresis and transferred to PVDF membrane (Millipore, USA, IPVH00010). After blocking with 5% skim milk powder at room temperature for 1 h, Bax (acbam, UK, ab32503), Bcl-2 (acbam, UK, ab182858), cleaved-caspase 3 (acbam, UK, ab32042), SOX2 (acbam, UK, ab97959), Oct4 (acbam, UK, ab19857), NANOG (acbam, UK, ab109250), γH2AX (acbam, UK, ab81299), p-ATM (Ser1981) (acbam, UK, ab308338), p-ATR (Ser428) (acbam, UK, ab316925), p-p53 (Ser15) (acbam, UK, ab223868), AMPK (acbam, UK, ab271188), p-AMPK (Thr172) (acbam, UK, ab133448), ACC (acbam, UK, ab223781), p-ACC (Ser79) (acbam, UK, ab256461), mTOR (acbam, UK, ab134903), p-mTOR (Ser2448) (acbam, UK, ab137133), and β-actin (acbam, UK, ab179467 ) antibodies were incubated at 4 °C overnight. The next day, the corresponding HRP-labeled secondary antibody was added, and the protein bands were detected with ECL colorimetric reagent (ThermoFisher Scientific, 32106) after incubation at room temperature for 1 h. The protein bands were quantified using ImageJ software, and the relative expression levels were calculated by normalizing to β-actin as the internal reference.

### Statistical analysis

The data are presented as mean ± SD, and statistical analyses were performed using GraphPad Prism 10.2 (GraphPad Software, San Diego, CA, USA). Differences between multiple groups were assessed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Statistical significance was considered at $p < 0.05$.

## Results

### Effect of GAA on GBC cell viability and colony formation ability

To evaluate the impact of GAA on GBC cell proliferation and its potential synergistic effect with DDP, we assessed cell viability and colony formation ability following treatment with GAA alone and in combination with DDP.

The chemical structure of GAA, as depicted in Fig. 1A, includes hydroxyl, carboxyl, and ketone groups, which contribute to its antioxidant, anti-inflammatory, and anti-tumor properties. Through these functional groups, GAA is capable of scavenging free radicals, inhibiting tumor cell proliferation, and modulating inflammatory pathways. In HGBEC, Fig. 1B shows that treatment with various concentrations of GAA had no significant effect on cell viability, except at 80 μM, where cell viability was notably reduced by 23.81% ($p < 0.05$). Conversely, in GBC-SD cells (Fig. 1C), GAA exerted a concentration-dependent inhibitory effect on cell viability, with significant reductions observed at concentrations of 5 μM and above, ranging from a 5.7% decrease to a 41.7% decrease ($p < 0.05$).

When GAA was combined with DDP (Fig. 1D), the IC50 value of DDP was significantly reduced from 8.98 μM to 4.07 μM ($p < 0.05$), indicating a potent synergistic effect. Among the treatment groups (Fig. 1E), cell viability was significantly higher in the Control group (untreated cells group) DPP compared to the other groups, with the DPP + GAA group showing the greatest inhibitory effect, resulting in a 42.4% decrease ($p < 0.05$ ). In the colony formation assay (Fig. 1F), the colony-forming ability of the DPP and GAA groups was significantly reduced compared to the untreated cells group. Notably, the DPP + GAA group demonstrated the most substantial decrease in colony formation, significantly lower than

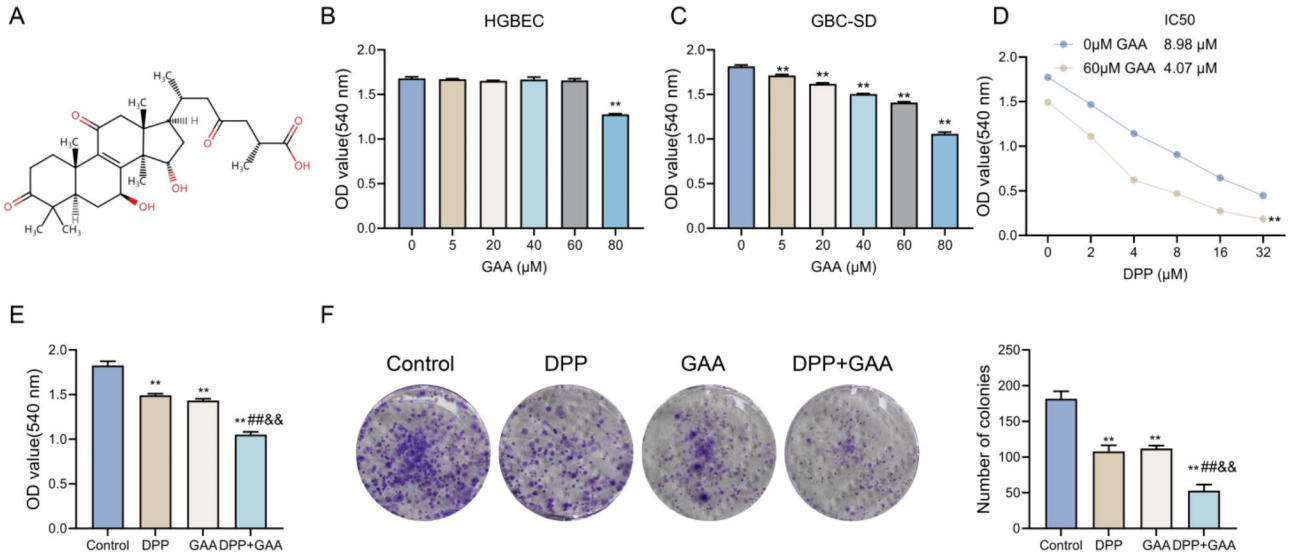


**Fig. 1** Effects of GAA on GBC-SD cell viability, DDP sensitivity, and colony formation ability
(**A**) Chemical structure of Ganoderic Acid A (GAA). (**B**) Cell viability of HGBEC cells after 24 h of treatment with various concentrations of GAA. (**C**) Cell viability of GBC-SD cells after 24 h of treatment with different concentrations of GAA. (**D**) Effect of GAA combined with cisplatin (DDP) on the IC50 value of GBC-SD cells. (**E**) Cell viability of GBC-SD cells treated with the Control group (untreated cells group), DPP group, GAA group, and DPP + GAA group. (F) Effect of different treatment groups on the colony formation ability of GBC-SD cells. $^{**}$, $P < 0.05$: Comparison with Control or 0 μM; $^{\#\#}$, $P < 0.05$: Comparison with DPP; $^{\&\&}$, $P < 0.05$: Comparison with GAA

both the DPP and GAA groups ($p < 0.05$). These results suggest that GAA effectively inhibits GBC cell viability and colony formation, while also enhancing the anticancer activity of DDP.

### Effects of GAA on GBC cell apoptosis

Further investigate the pro-apoptotic effects of GAA on GBC-SD cells, we conducted TUNEL and Western Blot assays to assess apoptosis-related markers following treatment with GAA alone and in combination with DDP.

The TUNEL assay results (Fig. 2A) demonstrated that, compared to the Control group (untreated cells group), the apoptosis rate was significantly increased in both the DPP and GAA groups ($p < 0.05$). The DPP + GAA combination treatment group exhibited the highest apoptosis rate at 38.5% (± 0.58 SD), which was significantly greater than that observed in either the DPP or GAA alone treatment groups ($p < 0.05$). Western Blot analysis (Fig. 2B) further confirmed these findings. In both the DPP and GAA groups, there was a significant increase in the expression of the pro-apoptotic protein Bax/Bcl-2 and cleaved-caspase 3, when compared to the untreated cells group ($p < 0.05$). In the DPP + GAA combination treatment group, the levels of Bax/Bcl-2 and cleaved-caspase 3 were significantly higher ($p < 0.05$).

Together, these results indicate that GAA promotes apoptosis in GBC cells by upregulating Bax/Bcl-2 and cleaved-caspase 3. The increased Bax/Bcl-2 ratio suggests activation of the mitochondrial apoptotic pathway [31]. The combination of GAA and DDP further enhances this pro-apoptotic effect, suggesting a synergistic impact on GBC cell apoptosis.

### Effects of GAA on GBC cell stemness

To assess the impact of GAA on the stemness of GBC-SD cells, we performed sphere formation assays, immunofluorescence detection, and Western Blot analysis, with particular focus on the effect of combined treatment with DDP.

The sphere formation assay (Fig. 3A) revealed that the sphere-forming ability of both the DPP and GAA groups was significantly reduced compared to the Control group ( untreated cells group). Notably, the DPP + GAA combination treatment led to a further decrease in sphere formation, indicating an enhanced inhibitory effect on cell stemness. Immunofluorescence detection of the stemness marker CD44 (Fig. 3B) showed that the fluorescence intensity of CD44 was significantly reduced in both the DPP and GAA groups ($p < 0.05$). The DPP + GAA group exhibited the lowest fluorescence intensity, significantly lower than that in the DPP or GAA groups alone ($p < 0.05$). Western Blot analysis (Fig. 3C) assessed the expression of key stemness markers, including SOX2, Oct4, and NANOG. Compared to the Control group, the expression levels of all three markers were significantly reduced in the DPP and GAA groups ($p < 0.05$). The DPP + GAA group demonstrated the most pronounced inhibition of these stemness markers, further highlighting the synergistic effect of the combination treatment.

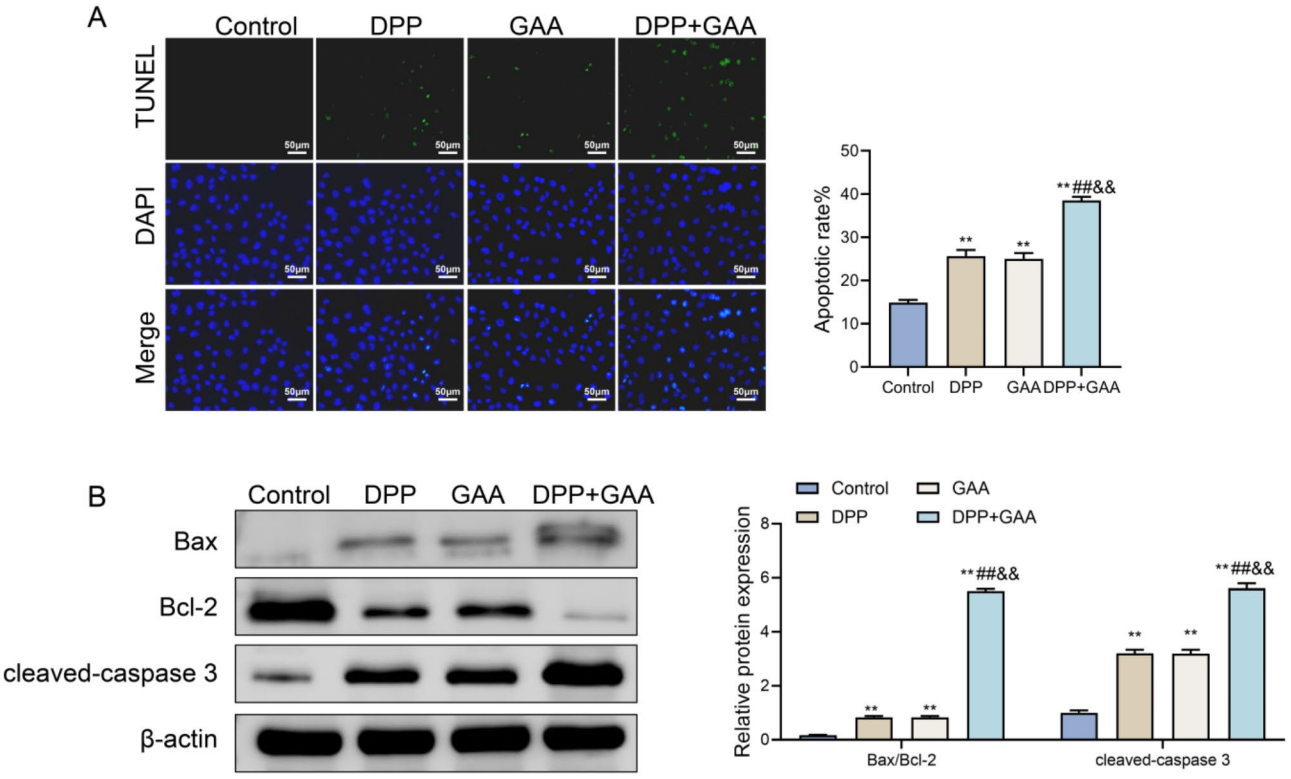


**Fig. 2** GAA enhances the anticancer effect of DDP by promoting apoptosis of GBC-SD cells
(**A**) TUNEL assay was used to detect apoptosis in gallbladder cancer cells. Green fluorescence represents apoptotic cells, while DAPI staining highlights the cell nuclei. The bar graph on the right shows the statistical analysis of the apoptosis rate for each group. (**B**) Western Blot analysis of the protein expression levels of Bax/Bcl-2, and cleaved-caspase 3. The bar graph on the right shows relative quantitative analysis of protein expression in each treatment group. $^{**}$, $P < 0.05$: Comparison with Control ( untreated cells group); $^{\#\#}$, $P < 0.05$: Comparison with DPP; $^{\&\&}$, $P < 0.05$: Comparison with GAA

These findings demonstrate that GAA significantly inhibits the stemness of GBC cells by downregulating key stemness markers such as CD44, SOX2, Oct4, and NANOG, which are well-established regulators of self-renewal, pluripotency, and therapeutic resistance in gallbladder cancer stem cells [32, 33]. Furthermore, GAA enhances the anticancer effects of DDP, supporting its potential as a therapeutic adjunct for targeting cancer stem cells in GBC.

#### Effects of GAA on DNA damage of GBC cells

To evaluate the effect of GAA on DNA damage in GBC cells, we used the comet assay to measure DNA damage levels and Western Blot analysis to assess the expression of key DNA damage-related proteins. This experiment was conducted to investigate the mechanisms underlying the effects of GAA alone and in combination with DDP.

The results from the comet assay (Fig. 4A) showed that compared to the Control group ( untreated cells group), both the DPP and GAA groups exhibited significantly increased DNA damage ($p < 0.05$). The DPP + GAA combination group demonstrated the highest level of DNA damage, as indicated by significantly increased tail DNA percentage and tail moment, which were used as the primary quantification metrics ($p < 0.05$), thereby indicating enhanced DNA damage. Western Blot analysis (Fig. 4B) revealed that the expression levels of key DNA damage markers, γH2AX, p-ATM, p-ATR and p-p53, were significantly elevated in both the DPP and GAA groups compared to the Control group ($p < 0.05$). Notably, the DPP + GAA combination group exhibited the highest expression of these markers ($p < 0.05$), indicating enhanced DNA damage.

These findings suggest that GAA promotes DNA damage in GBC cells, with a more pronounced effect when combined with DDP. The combination treatment significantly enhances the DNA damage response (DDR), as evidenced by increased expression of γH2AX, p-ATM, p-ATR, and p-p53. Since ATM and ATR are key regulators of the homologous recombination (HR) pathway, the upregulation of these proteins implies that GAA may sensitize GBC cells to chemotherapy by enhancing HR-mediated DNA damage repair signaling. This highlights the therapeutic potential of GAA in augmenting the efficacy of DDP via modulation of specific DDR pathways.

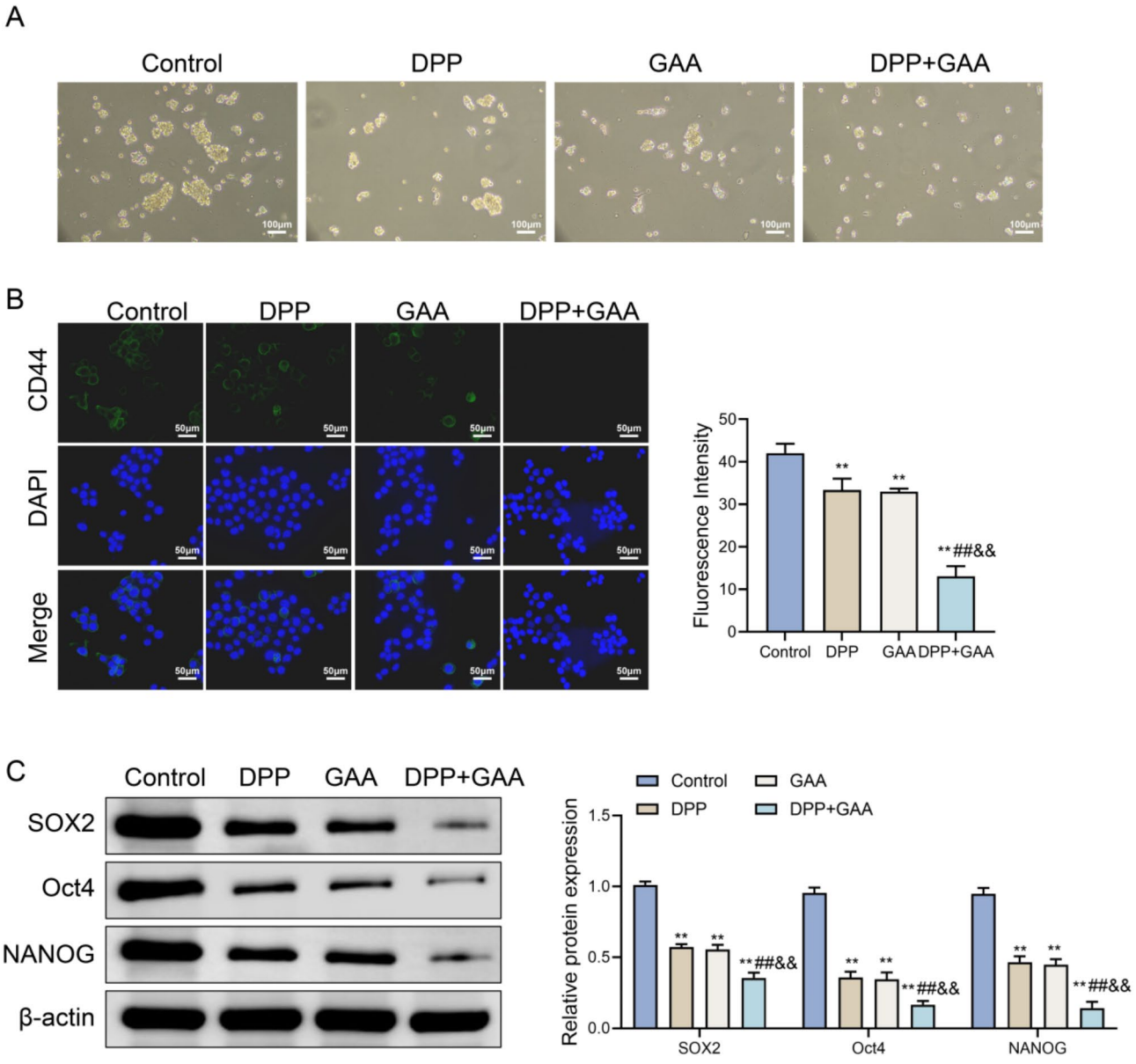


**Fig. 3** GAA inhibits stemness in GBC-SD cells, enhancing the anticancer effect of DDP
(**A**) Representative images showing sphere formation in GBC-SD cells treated with Control ( untreated cells group), DPP, GAA, or DPP + GAA. (**B**) Immunofluorescence detection of CD44 expression. Green fluorescence represents CD44, while DAPI staining highlights cell nuclei. The bar graph on the right shows the quantitative analysis of fluorescence intensity in each group. (**C**) Western Blot analysis of stemness-related proteins SOX2, Oct4, and NANOG. The bar graph on the right displays the relative protein expression levels for each group. $^{**}$, $P < 0.05$: Comparison with Control; $^{\#\#}$, $P < 0.05$: Comparison with DPP; $^{\&\&}$, $P < 0.05$: Comparison with GAA

### GAA regulates the AMPK/mTOR signaling pathway

Lastly, we evaluated the regulatory effects of GAA alone or in combination with DDP on the AMPK/mTOR signaling pathway by assessing the phosphorylation levels of AMPK, ACC, mTOR, and p-mTOR in GBC cells.

Western Blot analysis (Fig. 5) revealed that both the DPP and GAA groups significantly increased the phosphorylation of AMPK ($p < 0.05$), as well as the phosphorylation of its downstream target, ACC. In the DPP + GAA group, the upregulation of p-AMPK and p-ACC was even more pronounced, with levels significantly higher than in the single-treatment groups ($p < 0.05$). Furthermore, mTOR and its phosphorylated form, p-mTOR, were significantly down-regulated in both the DPP and GAA groups, with the DPP + GAA combination showing the most substantial reduction in p-mTOR expression ($p < 0.05$). These results suggest that GAA activates the AMPK pathway by enhancing the phosphorylation of AMPK and its downstream target, ACC, while inhibiting the mTOR pathway by reducing the expression of both

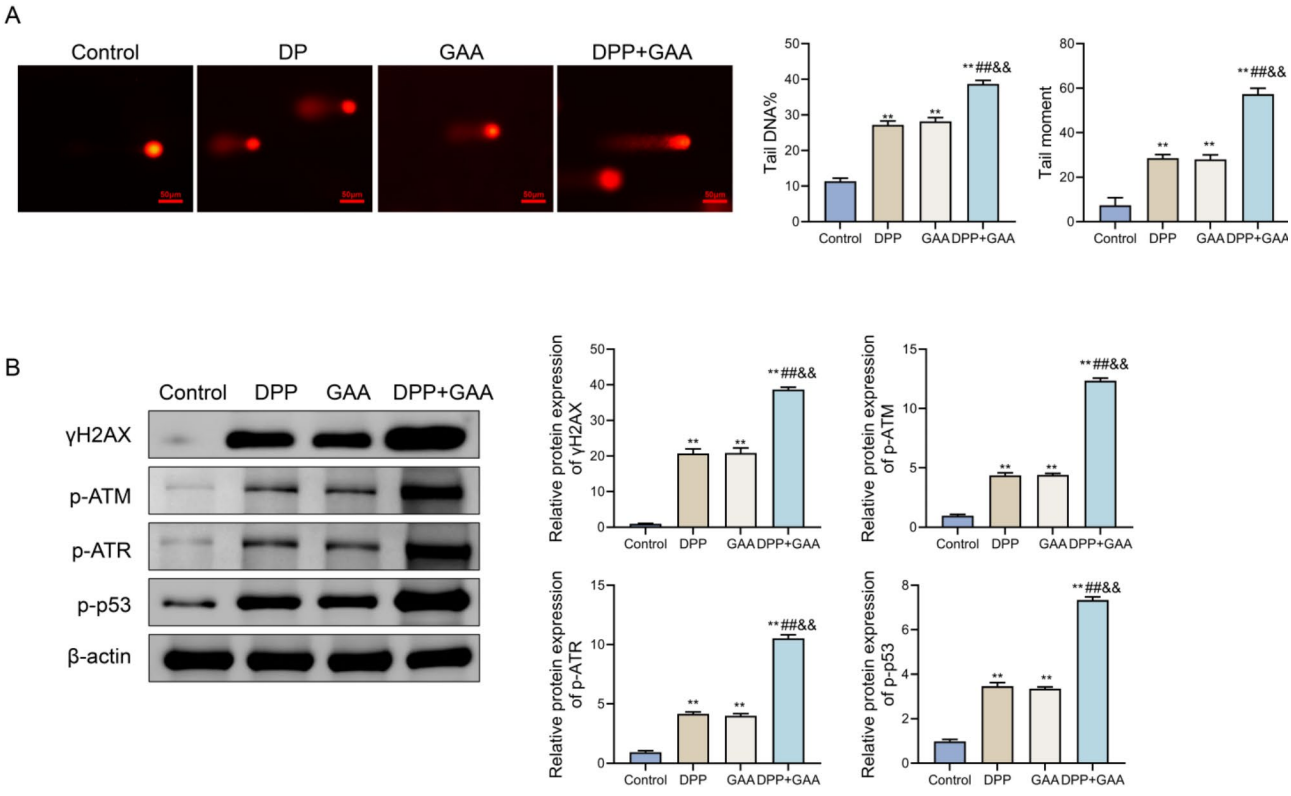


**Fig. 4** GAA enhances the anticancer effect of DDP by promoting DNA damage in GBC cells
(**A**) Comet assay showing DNA damage in gallbladder cancer cells treated with different groups. The images on the left display comet formation, while the bar graph on the right shows statistical analysis of tail DNA ratio and comet tail distance. (**B**) Western Blot analysis of the expression of DNA damage-related proteins γH2AX, p-ATM, p-ATR, and p-p53. The bar graph on the right presents the relative quantitative analysis of these proteins in each treatment group. $^{**}$, $P < 0.05$: Comparison with Control ( untreated cells group); $^{\#\#}$, $P < 0.05$: Comparison with DPP; $^{\&\&}$, $P < 0.05$: Comparison with GAA

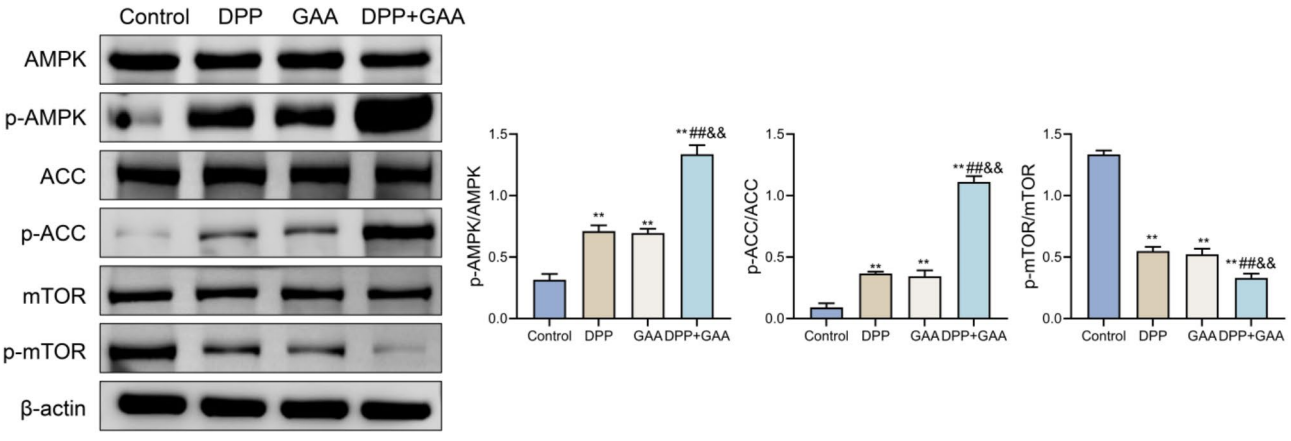


**Fig. 5** Western blot analysis of AMPK, p-AMPK, ACC, p-ACC, mTOR, and p-mTOR expression in GBC cells

mTOR and p-mTOR. Notably, when combined with DDP, GAA exerts a more potent regulatory effect on these signaling pathways, further reinforcing its potential to enhance the therapeutic efficacy of chemotherapy.

Western Blot analysis was performed to assess the expression levels of AMPK, p-AMPK, ACC, p-ACC, mTOR, and p-mTOR in gallbladder cancer cells treated with different groups. The bar graphs on the right display the relative quantitative analysis of these proteins in each treatment group. $^{**}$, $P < 0.05$: Comparison with Control ( untreated cells group); $^{\#\#}$, $P < 0.05$: Comparison with DPP; $^{\&\&}$, $P < 0.05$: Comparison with GAA.

## Discussion

This study systematically evaluated the anticancer effects of GAA in GBC cells and its potential to enhance the efficacy of DDP. The results showed that GAA, at a concentration of 60 μM, significantly inhibited cell proliferation, sphere-forming ability, and the expression of stemness-related markers (i.e., CD44, SOX2, Oct4, and NANOG) in GBC-SD cells. Furthermore, co-treatment with 60 μM GAA and 2 μM DDP markedly enhanced the inhibitory effects, suggesting a synergistic role in suppressing stemness and improving chemosensitivity. In terms of DNA damage, both GAA alone and in combination with DDP could significantly increase DNA fragmentation, as evidenced by comet assays showing higher tail DNA content and comet tail distance. Moreover, GAA upregulated key DNA damage response proteins, including γH2AX, p-ATM, p-ATR, and p-p53, indicating that GAA impairs the DNA damage repair process and sensitizes GBC cells to DDP-induced DNA damage. Additionally, GAA regulates cellular energy metabolism by activating the AMPK pathway and inhibiting the mTOR pathway, which disrupts cellular energy balance, further inhibiting cell growth and proliferation. This disruption of cellular metabolism, coupled with the enhanced DNA damage response, resulted in a more pronounced effect when GAA is used in combination with DDP, leading to increased apoptosis, inhibition of stemness, and enhanced DNA damage, thereby suggesting that GAA, by potentiating both DNA damage and stemness inhibition, could serve as an effective sensitizer to chemotherapy, thus improving the therapeutic outcomes of DDP in GBC cells.

The inhibition of cancer stemness is an important mechanism for overcoming chemoresistance, especially in cancers such as GBC, which are often resistant to chemotherapy [34]. Several studies have demonstrated that cancer stem cells (CSCs) play a significant role in mediating chemotherapy resistance by evading drug-induced apoptosis and promoting tumor recurrence [35]. In the context of GBC, targeting stemness has been suggested as a promising strategy to enhance the sensitivity of tumor cells to chemotherapy. Our research results show that GAA significantly inhibits the proliferation and stemness of GBC cells, especially in inhibiting the sphere-forming ability and the expression of stemness markers (such as CD44, SOX2, Oct4 and NANOG), showing strong anticancer effects Effect, consistent with previous studies showing that ganoderic acid compounds can reduce cancer stem cells by inhibiting stemness markers [36, 37]. Especially when used in combination with DDP, the inhibitory effect of GAA was found to be more obvious, further indicating that GAA can enhance the anticancer effect of DDP through synergy. In addition, GAA further enhanced its anticancer effect by promoting DNA damage and apoptosis of GBC cells. The results of our comet experiment showed that when GAA was treated alone or combined with DDP, the DNA fragmentation of GBC cells increased significantly, the tail DNA content and comet tail distance increased significantly, and the expression of γH2AX, p-ATM, p-ATR and p-p53 also increased accordingly. These findings indicate that GAA enhances the inhibitory effect of DNA damage repair by activating the DNA damage response signaling pathway, consistent with previous findings that ganoderma acid compounds promote DNA damage [38, 39]. In addition, our study also reveals for the first time the mechanism by which GAA can significantly enhance the effects of DNA damage and apoptosis when used in combination with DDP. It is worth noting that the regulatory effect of GAA on the AMPK/mTOR signaling pathway was also confirmed in this study. GAA significantly upregulated the phosphorylation levels of AMPK and ACC, while significantly inhibiting the expression of mTOR and its phosphorylated form. Activation of the AMPK pathway and inhibition of the mTOR pathway jointly promote the imbalance of cellular energy metabolism, thereby inhibiting the growth and proliferation of GBC cells. When used in combination with DDP, this regulatory effect is more significant, indicating that GAA cooperates with DDP to exert anticancer effects through dual mechanisms. Overall, our results suggest that GAA's ability to downregulate these stemness markers in GBC cells could potentially help overcome DDP resistance, thus improving treatment outcomes. However, these warrant further exploration in clinical settings to validate whether stemness inhibition by GAA can enhance the response to chemotherapy in chemoresistant GBC.

Given the recent results of the TOPAZ-1 trial [40], which demonstrated the efficacy of durvalumab in combination with chemotherapy for GBC, an interesting question arises regarding whether immune checkpoint inhibitors or other immune therapies could further augment the effect of GAA in vivo. In this study, we demonstrated that GAA enhances apoptosis in GBC cells, potentially leading to increased antigen presentation and immune activation. While the in vitro results of GAA suggest that it may promote cell death and DNA damage [41], we did not explore the immune response in this context. It is conceivable that, in an in vivo setting, the increased apoptosis induced by GAA could enhance the tumor's immunogenicity, potentially making the cancer cells more susceptible to immune-mediated elimination. Future studies could investigate the combined effect of GAA with immune checkpoint inhibitors like durvalumab to evaluate whether GAA enhances anti-tumor immunity and promotes a more robust immune response in GBC.

While our study demonstrates significant effects in GBC-SD cells, we acknowledge that the results from a single cell line may limit the generalizability of the findings to all GBC subtypes [42]. The biological diversity within GBCs, including differences in genetic mutations and stemness, may lead to varying responses to GAA and DDP treatment. Therefore, further studies should expand to include other GBC cell lines and explore whether these findings hold true across different models, such as patient-derived xenografts (PDXs) or genetically diverse cell lines. Additionally, the potential of GAA as an adjunct therapy should be tested in other cancers with known DDP resistance mechanisms, such as ovarian or lung cancer, to assess its broader applicability. Future studies could also integrate preclinical animal models to better predict the clinical efficacy and safety of GAA in combination with chemotherapy.

Although our study primarily focused on the role of GAA in enhancing DNA damage in combination with DDP, particularly through the upregulation of γH2AX, p-ATM, p-ATR and p-p53, additional molecular pathways involved in DNA damage response could be explored to provide a more comprehensive mechanistic understanding of how GAA sensitizes cancer cells to chemotherapy [41]. Specifically, pathways such as the ATM/Chk2 and ATR/Chk1 axis, DNA repair pathways like homologous recombination, and the involvement of DNA repair proteins such as BRCA1/2 could provide more clinical insights [43, 44]. Additionally, the role of GAA in modulating the balance between DNA repair and cell death pathways, such as the interplay between p53-mediated apoptosis and autophagy, should be addressed in future studies to reveal more detailed insights into how GAA enhances the response to DDP.

Moreover, although our study demonstrates the promising therapeutic effects of GAA in enhancing DDP sensitivity, it is important to acknowledge the potential side effects and toxicity associated with the combination treatment. The impact of GAA on normal tissues and its toxicity profile, particularly in combination with DDP, was not assessed in this study. Given that GAA affects key cellular processes such as DNA damage response, stemness inhibition, and metabolic pathways [45], the potential for off-target effects and toxicity in normal tissues cannot be overlooked. Previous studies have suggested that GAA has low toxicity to normal cells [46], but further in vivo studies assessing its safety, long-term toxicity, and organ-specific effects are warranted. The combination of GAA with DDP should also be evaluated for potential synergistic or antagonistic effects on healthy cells to determine if there is an increased risk of adverse effects such as immune suppression or organ damage. In light of the promising results from this study, future research should focus on determining the safety profile of GAA in combination with chemotherapy to ensure its clinical feasibility.

Herein, we determined that GAA can regulate the AMPK/mTOR signaling pathway by significantly upregulating the phosphorylation levels of AMPK and ACC, while inhibiting mTOR and its phosphorylated form. The activation of the AMPK pathway and inhibition of mTOR are key regulators of cellular energy metabolism, which disrupts energy homeostasis and inhibits tumor growth. This dual modulation of AMPK and mTOR suggests that GAA could have a potent anticancer effect by reprogramming the metabolic state of cancer cells, which often rely on altered energy metabolism for growth and survival. Notably, mTOR also plays a critical role in regulating cell cycle progression, particularly the transition from G1 to S phase. Inhibition of mTOR has been shown to induce G1 phase arrest by suppressing protein synthesis and growth signals necessary for cell cycle progression. In this study, the observed downregulation of mTOR and p-mTOR following GAA treatment raises the possibility that GAA may contribute to cell cycle arrest, further enhancing its anticancer effects [47, 48]. However, the downstream effects of AMPK/mTOR modulation are broad and could influence other cellular processes, including autophagy, nutrient sensing, and protein synthesis. The activation of AMPK may lead to the suppression of mTOR-dependent protein synthesis and may also induce autophagic processes, which could either promote or hinder cell survival, depending on the context. Future studies may investigate the broader effects of AMPK/mTOR modulation by GAA on autophagy, cellular stress response, and nutrient sensing pathways. Understanding these effects could further elucidate the mechanism through which GAA enhances DDP efficacy and provide insights into its potential as a metabolic modulator in cancer therapy. It is also important to note that our study did not directly evaluate metabolic parameters such as ATP levels, glucose uptake, or mitochondrial function, which limits mechanistic interpretation. Further research is warranted to elucidate these downstream metabolic effects.

While this study demonstrates the promising anticancer effects of GAA in vitro, several challenges must be considered when translating these findings to in vivo models. First, we did not perform a formal synergy analysis such as combination index (CI) or isobologram analysis to quantitatively assess synergy and could provide a more precise evaluation of the interaction between GAA and DDP. Second, the pharmacokinetics of GAA should be explored as this could influence its efficacy and safety when administered systemically. Specifically, GAA's bioavailability, distribution, and metabolism may differ significantly in vivo, potentially affecting its therapeutic outcomes, especially when combined with DDP. Additionally, the safety of GAA, particularly in combination

with chemotherapy, remains unknown, and concerns regarding potential toxicity, both acute and long-term, need to be carefully addressed. Although the cytotoxicity of GAA was evaluated in HGBEC cells as a normal control, its effects on other non-malignant cell types—especially hepatocytes and renal cells—remain unclear. Since liver and kidney toxicity are key considerations in systemic drug development, further studies should investigate GAA's potential off-target effects on major organs. The effects of GAA on different GBC subtypes and potential organ toxicity require further investigation. Moreover, whether GAA exerts comparable antitumor effects in animal models or patient-derived xenograft (PDX) models remains unclear and should be addressed in future studies to evaluate its clinical translatability. To clarify its clinical applicability, future studies should focus on assessing the pharmacokinetics, long-term safety, and organ-specific toxicities of GAA in vivo, particularly in combination with DDP, to evaluate its potential as an adjunct in cancer therapy.

## Conclusion

This study showed that GAA significantly inhibited the proliferation, stemness and apoptosis of GBC cells and enhanced the anticancer effects of DDP through DNA damage response. The combined use of GAA and DDP was found to significantly improve the therapeutic effects, suggesting GAA potential clinical application value as an adjuvant therapeutic drug.

## Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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**Author contributions**
G.Z.: Conceptualization; Data curation; Formal analysis; Investigation; Visualization; Writing - original draft; Writing - review & editing. H.L.: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. J.W.: Data curation; Formal analysis; Investigation; Methodology; Resources; Writing - review & editing. X.S.: Formal analysis; Methodology; Resources; Visualization; Writing - review & editing. L.H.: Data curation; Methodology; Writing - review & editing. M.Z.: Data curation; Formal analysis; Writing - review & editing. J.H.: Conceptualization; Formal analysis; Supervision; Writing - review & editing.

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**Data availability**
The data used to support the findings of this study are available from the corresponding author upon request.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
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**Competing interests**
The authors declare no competing interests.

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