# Gambogic acid potentiates the chemosensitivity of colorectal cancer cells to 5-fluorouracil by inhibiting proliferation and inducing apoptosis

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**Abstract.** Chemotherapy using 5-fluorouracil (5-FU) for colorectal cancer (CRC) has low specificity and response rates, leading to severe side effects. Gambogic acid (GA), a traditional Chinese medicine, has multi-targeted anticancer effects, including growth inhibition and apoptosis induction. However, it is unclear whether a combination of 5-FU and GA has synergistic anticancer effects in CRC cells. In this study, SW480 and HCT116 human CRC cells and human intestinal epithelial cells (IECs) were treated with different concentrations of 5-FU, GA or 5-FU+GA. A Cell Counting kit-8 assay was conducted to quantify cell proliferation. The combination index (CI) was calculated and the median-effect principle was applied to analyze the interaction between 5-FU and GA. Flow cytometry was used to determine the percentage of cells undergoing apoptosis. Reverse transcription-quantitative polymerase chain reaction and western blotting were applied to measure P53, survivin and thymidylate synthase (TS) mRNA and protein levels. It was found that 5-FU+GA more pronouncedly inhibited cell growth and induced apoptosis, compared with either monotherapy. CI values <1 indicated the synergistic effects of the drugs. 5-FU+GA further decreased P53, survivin and TS mRNA and protein levels in the two CRC cell lines compared with single drugs, whereas increased P53 protein levels were observed in HCT116 cells. Moreover, 5-FU+GA did not increase cytotoxicity to IECs. These results demonstrate that GA enhances the anticancer effects of 5-FU on CRC cells. Combined treatment with 5-FU and GA is effective and safe for CRC cells, and may become a promising chemotherapy treatment.

## Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies and a leading cause of cancer-related mortality worldwide (1). China has a high incidence of CRC; with 149,722 estimated mortalities in 2011, it represents one-fifth of all cancers in terms of both morbidity and mortality (2).

Treatment options for CRC include surgery, chemotherapy and radiotherapy. Surgical resection is the main and most effective option (3). However, surgery alone is insufficient to treat CRC, and certain patients do not have the opportunity to undergo surgical treatment (4). Systemic chemotherapy is an important tool, particularly for patients with advanced CRC: it can decrease clinical symptoms, improve the quality of life, and prolong survival (5).

5-Fluorouracil (5-FU)-based chemotherapeutics are commonly used in CRC (6), with a single drug response rate of ~21% (7). Clinicians have used oxaliplatin (4) or irinotecan (8) in combination with 5-FU to enhance chemotherapeutic effects, but have only obtained response rates of 30-50% (4). In addition, these drugs have low specificity; although they increase 5-FU activity, they also promote damage to normal cells, and side effects are prominent (9).

Therefore, the development of novel chemotherapy strategies is essential. Several studies (10-12) have shown that combining chemotherapeutics with traditional Chinese medicine can result in cooperative effects and enable the required doses of chemotherapeutics to be reduced, resulting in lower drug toxicity, decreased side effects and reduced drug

resistance. This has become a topic of particular research interest in cancer treatment.

Gambogic acid (GA), a common traditional Chinese medicine and the main active component of *Garcinia hanburyi*, has limited side effects (13). Previous studies have shown that GA has multi-targeted antitumor effects, including inhibition of proliferation (14), induction of apoptosis (15), cell cycle arrest (16), and inhibition of angiogenesis (17), invasion and metastasis (18).

P53 is a key tumor suppressor (19); it induces apoptosis, which is one of the mechanisms that stop cancer cells from growing (20). Survivin, the smallest member of the inhibitor of apoptosis (IAP) family (21), is known to be a key regulator in cancer proliferation and apoptosis (22). The overexpression of survivin has been found to be associated with a poor prognosis (23) and chemoresistance (24). Thymidylate synthase (TS) is a key enzyme in 5-FU metabolism (25) and has been shown to function as an oncogene (26). 5-FU exerts cytotoxicity by inhibiting TS activity and interrupting DNA and RNA syntheses (27). High TS expression is also associated with 5-FU resistance (28) and poor prognosis (29).

So far, to the best of our knowledge, whether GA enhances 5-FU chemotherapy in CRC has not been investigated. In the present study, the effects of 5-FU combined with GA were evaluated in two CRC cell lines, and the effects of the combination on the regulation of P53, survivin and TS, apoptosis and chemoresistance-related genes were explored.

## Materials and methods

*Materials*. SW480 and HCT116 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) at 37°C with 5% $CO_2$. Human intestinal epithelial cells (IECs) were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS at 37°C with 5% $CO_2$. GA was obtained from Sigma-Aldrich (St. Louis, MO, USA); 5-FU was purchased from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China; H31020593). Cell counting kit-8 (CCK-8; C0037) was obtained from Beyotime Institute of Biotechnology (Haimen, China). Alexa Fluor®488 Annexin V/Dead Cell Apoptosis kit (V13245) was from Invitrogen (Thermo Fisher Scientific, Inc.). The antibodies against P53 (ab131442; rabbit polyclonal, 53 kDa), survivin (ab24479; rabbit monoclonal, 16 kDa), thymidylate synthase (ab3145; mouse monoclonal, 35 kDa) and β-actin (ab6276; mouse monoclonal, 42 kDa) were from Abcam (Cambridge, MA, USA).

*Cell proliferation assay*. SW480 or HCT116 cells, or IECs ($4x10^4$ cells/ml) were seeded in 96-well plates and cultured overnight. Solutions (100 µl) containing different concentrations of GA (0, 0.25, 0.5, 0.75, 1, 1.5, 2 and 3 µM), 5-FU (0, 6.25, 12.5, 25, 50, 100 and 200 µM) or 5-FU+GA were added for 48 h. Afterwards, 10 µl CCK-8 solution was added to each well, and the absorbance at 450 nm was read using a microplate reader (iMark; Bio-Rad Laboratories, Inc., Hercules, CA,

USA) after 2 h of incubation. All assays were carried out in triplicate.

*Combined effect analysis*. The interactions of the two drugs were evaluated by the median-effect principle, using the combination index (CI) method (30). CI values of 5-FU and GA were calculated using CompuSyn (USA) software (ComboSyn, Inc., Paramus, NJ, USA) where CI<1, CI=1 and CI>1 indicate synergism, addition and antagonism, respectively.

*Morphological observations*. SW480 or HCT116 cells were cultured in 25-cm$^2$ flasks to approximately ~80% confluency, and 5-FU (SW480, 122.14 µM; HCT116, 18.43 µM), GA (SW480, 0.75 µM; HCT116, 1 µM) and 5-FU+GA, respectively, were added for 48 h. After three washes, cells were assessed using an inverted optical microscope.

*Assessment of cell apoptosis*. Cells were harvested after 48 h incubation with GA, 5-FU or 5-FU+GA, and resuspended in Annexin-binding buffer to $2x10^6$ cells/ml. Annexin V and propidium iodide (PI) working solutions were added and the cells were incubated at room temperature for 15 min. Flow cytometry was performed (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed using FlowJo 7.6 software (FlowJo, LLC, Ashland, OR, USA). All assays were run in triplicate.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was extracted from the cells using Tripure isolation reagent (Roche Applied Science, Basel, Switzerland), and RNA samples were treated with DNase (Ambion; Thermo Fisher Scientific, Inc.). cDNA was synthesized from the obtained RNA samples using the Primescript 1st strand cDNA Synthesis kit (6110A; Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. Primers were designed using Primer Premier 6.0 software (Premier Biosoft, Palo Alto, CA, USA) and synthesized by Guangzhou Dahui Biotech Co., Ltd. (Guangzhou, China), with the following sequences (5' to 3'): Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; amplicon size, 164 bp), forward: TCCACTGGCGTCTTCACCACCAT and reverse: GGAGGCATTGCTGATGATCTTGAGG; P53 (amplicon size, 101 bp), forward: TGTTGGTCGGTGGGT TGGTAGT and reverse: GAGGTTGTCAGACAGGGT TTGGC; survivin (amplicon size, 133 bp), forward: AGC CCTTTCTCAAGGACCACCG and reverse: GCCAAGTCT GGCTCGTTCTCAG; TS (amplicon size, 101 bp), forward: CCATGCCCTCTGCCAGTTCTATG and reverse: TGGCGATGTTGAAAGGCACACC. qPCR was carried out using a SYBR Green Realtime PCR Master Mix kit [E090; Novo Protein Scientific (Shanghai), Inc., Shanghai, China] with a reaction mixture containing 2 µl cDNA, 0.25 µl each primer and 10 µl SYBR Green at 95°C (5 min) followed by 45 cycles of 95°C (10 sec) and 60°C (30 sec). All assays were run in triplicate. CT values were assessed using IQ5 software (Bio-Rad Laboratories, Inc.). Relative expression of target genes was determined using the $2^{-\Delta\Delta Cq}$ method (31).

*Western blot analysis*. Total protein was extracted from the cells with SDS-PAGE protein sample buffer (Beyotime Institute of Biotechnology, Haimen, China), resolved by SDS-PAGE
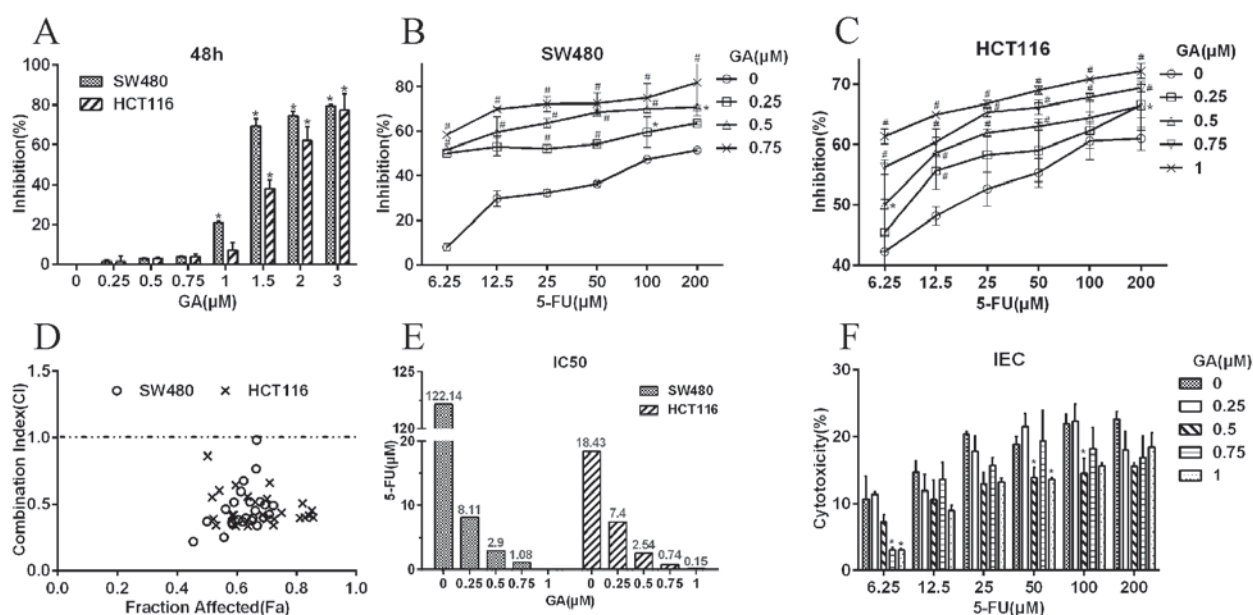
Figure 1. Effects of 5-FU combined with GA. (A) Inhibitory effects of various GA concentrations on SW480 and HCT116 cells at 48 h. *P<0.05 vs. control (0 $\mu$M). (B and C) Combined effects of GA and 5-FU on (B) SW480 and (C) HCT116 cells. Cells were co-exposed to different concentrations of 5-FU and non-inhibitory concentrations of GA for 48 h. *P<0.05 and #P<0.01 vs. 5-FU alone. (D) Combination index (CI) plot for GA+5-FU treatment. The points below the line indicate CI<1. (E) $IC_{50}$ values of 5-FU when combined with GA at different concentrations. (F) Cytotoxicity of 5-FU+GA on IECs at 48 h. *P<0.05 vs. 5-FU alone. All assays were run in triplicate. 5-FU, 5-fluorouracil; GA, gambogic acid; $IC_{50}$, 50% cell growth inhibitory concentration; IEC, intestinal epithelial cell.

(concentration gel, 5%; separation gel, 10%) and transferred onto polyvinylidene fluoride membranes. After blocking with 5% non-fat milk, membranes were incubated with anti-P53 (1:1,000), anti-survivin (1:1,000), anti-TS (1:100) and anti-$\beta$-actin (1:5,000) antibodies at 4°C overnight. This was followed by incubation with goat anti-rabbit antibody (1:10,000; SA00001-2;) or goat anti-mouse antibody (1:10,000; SA00001-1; both Wuhan Sanying, Biotechnology, Wuhan, China) at room temperature for 1 h. Signals were visualized with the SuperSignal West PICO chemiluminescent detection system (Pierce; Thermo Fisher Scientific, Inc.). Protein bands were detected using Quantity One version 4.62 software (Bio-Rad Laboratories, Inc.) and relative protein levels were calculated based on $\beta$-actin protein. All assays were run in triplicate.

*Statistical analysis*. Data are presented as the mean ± standard deviation. Comparisons were performed by one-way analysis of variance using SPSS version 21.0 software (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

*5-FU and GA display synergistic inhibitory effects*. As shown in Fig. 1A, GA at 0.25, 0.5 and 0.75 $\mu$M did not inhibit the proliferation of SW480 cells, and at 0.25, 0.5, 0.75 and 1 $\mu$M did not inhibit the proliferation of HCT116 cells (P>0.05). However, GA at higher concentrations inhibited cell growth in a concentration-dependent manner (P<0.05). To avoid the inhibitory effects of GA, non-inhibitory GA concentrations were selected for further combination experiments, that is, 0.25, 0.5 and 0.75 $\mu$M for SW480 cells and 0.25, 0.5, 0.75 and 1 $\mu$M for HCT116 cells.

Notably, 5-FU+GA exhibited a more pronounced inhibitory effect compared with 5-FU monotherapy (Fig. 1B and C). The inhibitory effects increased as the GA concentration in the combination increased, indicating that GA enhanced 5-FU-induced inhibition in a concentration-dependent manner (P<0.05).

All CI values were <1 for treatment with 5-FU (6.25, 12.5, 25, 50, 100 and 200 $\mu$M) combined with GA (0.25, 0.5 and 0.75 $\mu$M for SW480 cells; 0.25, 0.5, 0.75 and 1 $\mu$M for HCT116 cells) at 48 h, suggesting that the two drugs in these concentrations function synergistically (Fig. 1D).

Based on the above data, the 50% cell growth inhibitory concentrations ($IC_{50}$ values) of 5-FU were calculated (Fig. 1E). The $IC_{50}$ decreased with increasing concentration of GA in the combination, which directly demonstrated that GA enhanced the sensitivity to 5-FU of SW480 and HCT116 cells, in a concentration-dependent manner.

As GA was able to potentiate 5-FU cytotoxicity in cancer cells, whether 5-FU+GA affects normal cells in a similar manner was further investigated. As shown in Fig. 1F, no significant increase in cytotoxicity was observed. Instead, GA decreased the cytotoxicity of 5-FU in IECs (P<0.05).

To further explore these effects of 5-FU+GA, maximum non-inhibitory GA concentrations were selected in subsequent experiments, that is, 0.75 $\mu$M for SW480 cells and 1 $\mu$M for HCT116 cells. $IC_{50}$ values were selected as the 5-FU concentrations, that is, 122.14 and 18.43 $\mu$M for SW480 and HCT116 cells, respectively.

*Morphological changes of cells*. SW480 and HCT116 cells in the control group were adherent, with clear bar-shaped outlines, and good refraction. Following treatment with GA for 48 h, cell numbers decreased and cell gaps widened. In
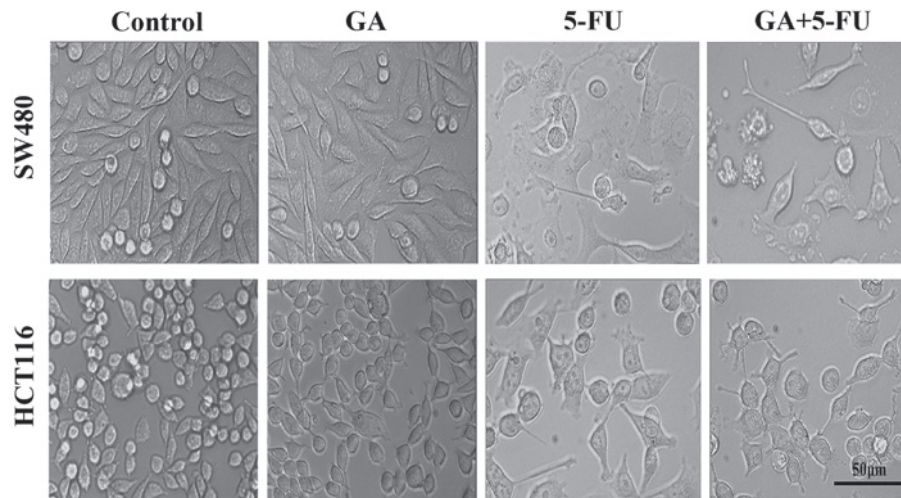
Figure 2. Morphological changes in cells treated with 5-FU, GA or 5-FU+GA. Cells were treated with GA (SW480, 0.75 $\mu$M; HCT116, 1 $\mu$M), 5-FU (SW480, 122.14 $\mu$M; HCT116, 18.43 $\mu$M) or GA+5-FU for 48 h. Untreated cells were used as controls. 5-FU, 5-fluorouracil; GA, gambogic acid.
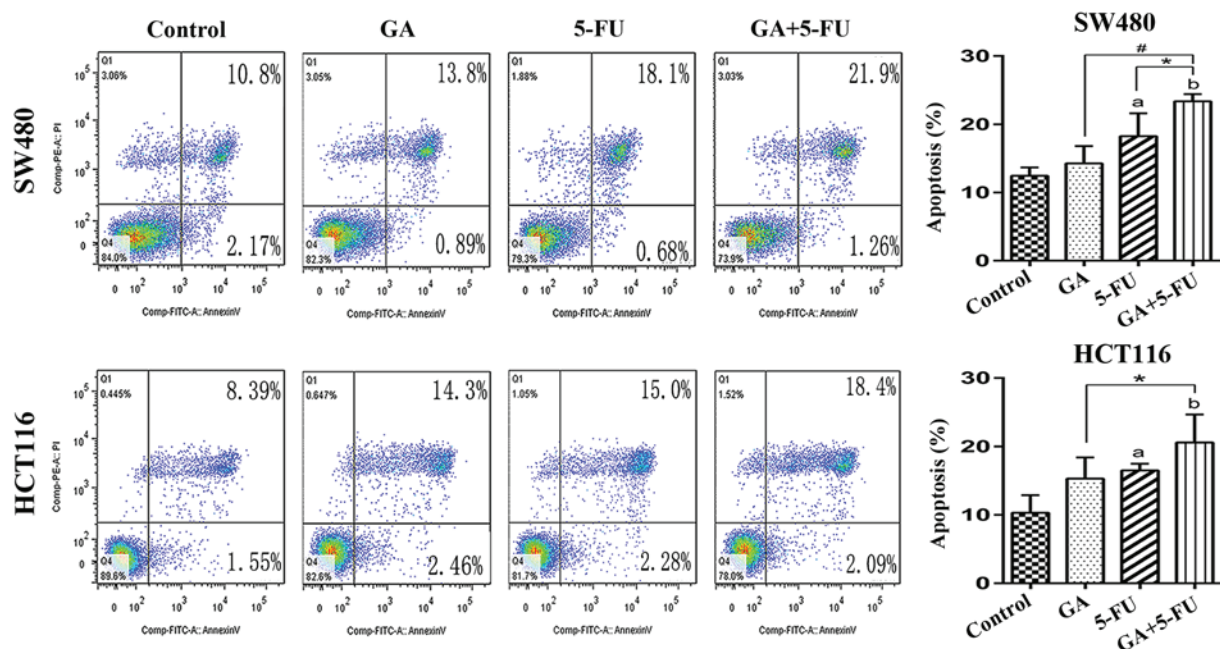


Figure 3. Apoptosis in cells treated with 5-FU, GA or 5-FU+GA. Cells were treated with GA (SW480, 0.75 $\mu$M; HCT116, 1 $\mu$M), 5-FU (SW480, 122.14 $\mu$M; HCT116, 18.43 $\mu$M) or GA+5-FU for 48 h. Untreated cells were used as controls. [a]P<0.05 and [b]P<0.01 vs. the control. [*]P<0.05 and [#]P<0.01. All assays were run in triplicate. 5-FU, 5-fluorouracil; GA, gambogic acid; PE, phytoerythrin; PI, propidium iodide; FITC, fluorescein isothiocyanate.

the 5-FU group, cell numbers decreased greatly; cells became round with shrunken bodies and obscure outlines. The effects were more pronounced in the combination group (Fig. 2).

*GA enhances 5-FU-induced apoptosis.* Apoptosis rates were increased in SW480 and HCT116 cells treated with 5-FU alone for 48 h compared with the respective control values (P<0.05); the combination yielded stronger effects compared with 5-FU or GA alone (P<0.05). These findings indicate that the synergistic inhibition of 5-FU+GA partly resulted from increased apoptosis (Fig. 3).

*Effects on P53, survivin and TS mRNA levels.* To further explore the synergistic effects of 5-FU and GA, P53, survivin

and TS mRNA expression levels were examined. 5-FU alone decreased P53, survivin and TS mRNA levels in SW480 cells compared with those in the control group; it also decreased P53 and TS mRNA levels in HCT116 cells (P<0.01). GA alone decreased survivin mRNA levels in SW480 cells and decreased P53, survivin and TS mRNA levels in HCT116 cells (P<0.05). The effects of the combination were more pronounced in both SW480 and HCT116 cells compared with the effects of 5-FU or GA alone (P<0.05; Fig. 4).

*Effects on P53, survivin and TS protein levels.* Compared with control group levels, 5-FU alone decreased P53, survivin and TS protein levels in SW480 cells, whereas in HCT116 cells 5-FU alone increased P53 protein levels and decreased
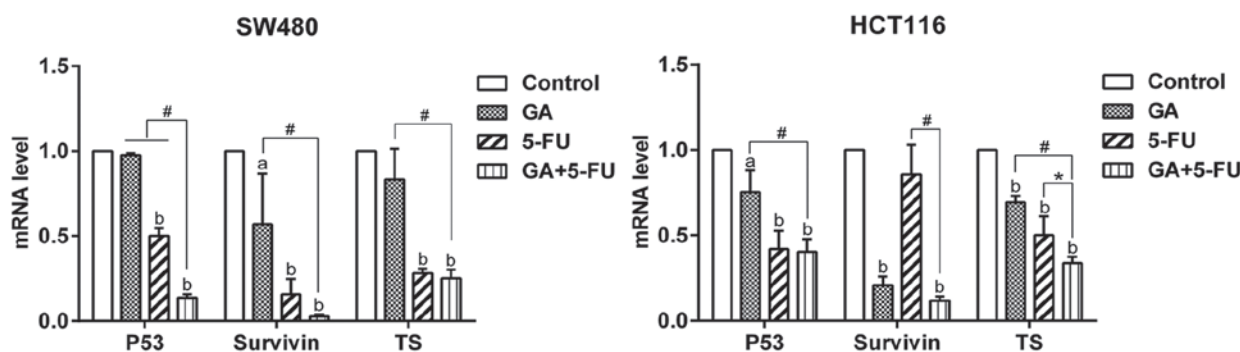
Figure 4. Changes of P53, survivin and TS mRNA levels. Cells were treated with GA (SW480, 0.75 $\mu$M; HCT116, 1 $\mu$M), 5-FU (SW480, 122.14 $\mu$M; HCT116, 18.43 $\mu$M) or GA+5-FU for 48 h. Untreated cells were used as controls. [a]$P<0.05$ and [b]$P<0.01$ vs. the control. [*]$P<0.05$ and [#]$P<0.01$. All assays were run in triplicate. 5-FU, 5-fluorouracil; GA, gambogic acid.
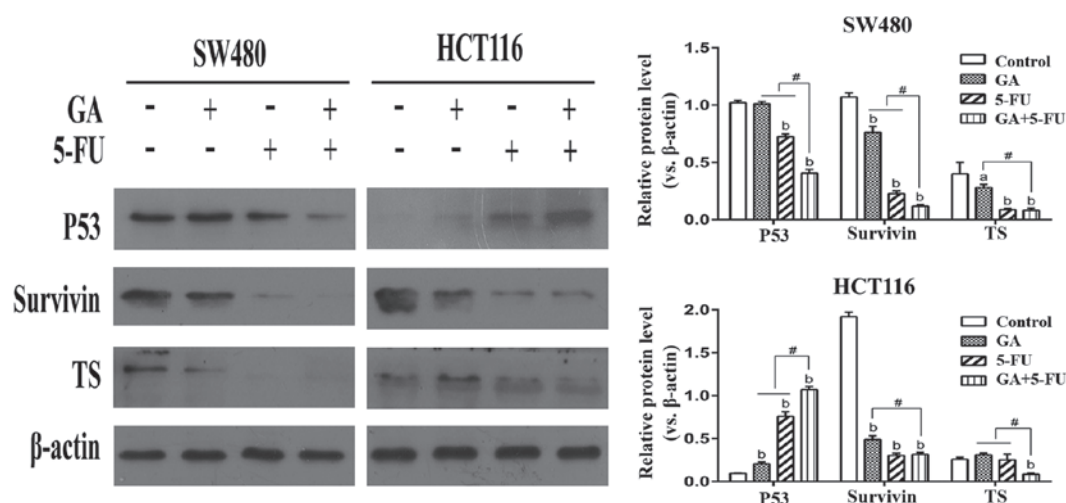


Figure 5. Changes of P53, survivin and TS protein levels. Cells were treated with GA (SW480, 0.75 $\mu$M; HCT116, 1 $\mu$M), 5-FU (SW480, 122.14 $\mu$M; HCT116, 18.43 $\mu$M) or GA+5-FU for 48 h. Untreated cells were used as controls. [a]$P<0.05$ and [b]$P<0.01$ vs. the control. [#]$P<0.01$. All assays were run in triplicate. 5-FU, 5-fluorouracil; GA, gambogic acid.

survivin protein levels (P<0.01). GA alone decreased survivin and TS protein levels in SW480 cells (P<0.05); GA alone increased P53 protein levels and decreased survivin protein levels in HCT116 cells (P<0.01).

In comparison with 5-FU or GA alone, 5-FU+GA further decreased P53, survivin and TS protein levels in SW480 cells, and further decreased survivin and TS protein levels in HCT116 cells (P<0.01). P53 protein levels in HCT116 cells were increased to a greater extent by 5-FU+GA than by either 5-FU or GA alone (P<0.01; Fig. 5).

## Discussion

GA is a novel anticancer drug whose mechanisms have not been fully explored. Wang *et al* (11) found that 5-FU and GA have synergistic effects in gastric cancer, both *in vitro* and *in vivo*; the proposed mechanism involved TS, dihydropyrimidine dehydrogenase and orotate phosphoribosyltransferase regulation, elevated caspase-3 and poly (ADP-ribose) polymerase cleavage activity, and a decreased Bcl-2/Bax ratio.

In the present study, it was found that GA potentiated the cytotoxicity of 5-FU to SW480 and HCT116 cells in a concentration-dependent manner. 5-FU and GA together had

synergistic effects, and could further induce apoptosis. The synergism was also found in the regulation of P53, survivin and TS, at the gene and protein levels. Moreover, the combination of 5-FU and GA did not increase cytotoxicity to normal cells, indicating that the combination was not only effective, but also safe.

P53 is the most frequently mutated gene in human cancers (32), with mutations occurring in 40-50% of CRC patients (33). Mutant P53 not only loses tumor suppressive functions of the wild-type protein, but also gains new oncogenic activities, including promotion of growth, angiogenesis and metastasis, a change described as gain-of-function (34,35). A previous study has shown that patients with CRC and mutant P53 have increased chemoresistance and poorer prognosis compared with those harboring wild-type P53 protein (36).

The two CRC cell lines assessed in the present study have different P53 types: SW480 has mutant P53 (37) while HCT116 has wild-type (38). The results demonstrated that HCT116 cells were more sensitive to 5-FU than were SW480 cells; the IC$_{50}$ of 5-FU alone was 122.14 $\mu$M for SW480, nearly 7-fold that obtained for HCT116 cells (18.43 $\mu$M). This important difference in sensitivity may partly result from their P53 types, as mutant P53 is associated with chemoresistance (36).

When 5-FU was combined with GA, both types of cell exhibited enhanced sensitivity and the synergistic effects on SW480 were more pronounced. When combined with 0.25 $\mu$M GA, the $IC_{50}$ of 5-FU was only 8.11 $\mu$M, indicating a 15-fold reduction compared with the value obtained with 5-FU alone. The $IC_{50}$ decreased in accordance with increasing GA concentration in the combination. When combined with GA, SW480 cells were almost as sensitive to 5-FU as HCT116 cells were. Therefore, GA is able to reduce the therapeutically required dose of 5-FU, especially for SW480 cells.

The results of the present study showed that GA or 5-FU alone decreased SW480 mutant P53 mRNA and protein levels, and the combination resulted in more pronounced effects. In HCT116 cells, the combination of 5-FU and GA further increased wild-type P53 protein levels but decreased P53 mRNA expression. The decreased gene expression in HCT116 might be associated with negative-feedback inhibition.

P53 and survivin are both closely associated with apoptosis: Wild-type P53 induces apoptosis (20) while mutant P53 and survivin inhibit apoptosis (22,34). The results of the present study indicate that the combination of 5-FU and GA induced apoptosis more strongly than did either monotherapy, with regulation of P53 and survivin also being observed, suggesting that P53 and survivin-related apoptosis might be involved in the synergistic anticancer effect.

TS plays an important role in 5-FU metabolism and it is an important target of 5-FU chemotherapy (25). In the present study, combined 5-FU and GA treatment further decreased the mRNA and protein expression of TS, suggesting TS was also involved in the synergistic anticancer effect.

High expression levels of survivin and TS are associated with chemoresistance (24,28). In the present study, it was found that combined 5-FU and GA treatment further decreased the RNA and protein levels of survivin and TS compared with either monotherapy, consistent with improved chemoresistance and enhanced anticancer effects.

Overall, these findings demonstrate that GA potentiates the chemosensitivity of CRC cells to 5-FU without increasing the cytotoxicity to normal cells. Thus, this combination might provide a promising treatment for patients with CRC. Future studies are essential to evaluate this combination in animal models and explore the underlying mechanisms.

## Acknowledgements

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