### ORIGINAL ARTICLE \_\_

### Synergistic enhancement of the antitumor activity of 5-fluorouracil by bornyl acetate in SGC-7901 human gastric cancer cells and the determination of the underlying mechanism of action

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### Summary

**Purpose:** To investigate the anticancer activity of bornyl acetate and its combination with low dose 5-fluorouracil (5-FU) in human gastric cancer (SGC-7901) cells and to evaluate their effects on cell cycle, apoptosis, cancer cell morphology and DNA fragmentation.

**Methods:** The anticancer activity of bornyl acetate, 5-FU and their combination against human gastric cancer (SGC-7901) cells was evaluated by MTT assay. Flow cytometry using propidium iodide (PI) as a staining agent was used to study the effect of the extract on cell cycle phase distribution. Apoptosis induced by bornyl acetate and 5-FU was evaluated by Annexin V binding assay using flow cytometer. Alterations in cell morphology following apoptosis was studied by fluorescence microscopy as well as transmission electron microscopy.

**Results:** Bornyl acetate induced dose-dependent growth inhibitory effects on human gastric cancer cells in vitro. The combination of bornyl acetate with 5-FU induced a much more growth inhibitory effect on these cells indicating a



synergistic enhancement of anticancer activity of 5-FU. The combined effect of bornyl acetate and 5-FU also resulted in greater apoptosis induction as well as cell cycle arrest in comparison to the individual treatment by bornyl acetate or 5-FU. Fluorescence microscopy as well as transmission electron microscopy also revealed that the combination of bornyl acetate with 5-FU resulted in greater apoptosis induction as well as cell morphology alterations. The percentages of early as well as late apoptotic cells were much higher in the combination treatment in comparison to separate treatment by bornyl acetate or 5-FU.

**Conclusion:** Bornyl acetate potentiates the anticancer activity of 5-FU in human gastric cancer (SGC-7901) cells by inducing apoptosis, DNA fragmentation as well as G2/M cell cycle arrest.

*Key words:* apoptosis, bornyl acetate, cell cycle, flow cytometry, gastric cancer

### Introduction

Gastric cancer is the second foremost cause of cancer related deaths after lung cancer, although the global incidence rate has now decreased. Despite the reduced incidence of gastric cancer worldwide, this disease remains a predominant problem in many Asian countries in comparison to the Western countries. China is the country with the highest occurrence of gastric cancer, with a projected figure of 370,000 new cases each year, accounting for more than 40% of the worldwide annual cancer incidence. In China, gastric cancer represents the third leading cause of cancer related deaths [1,2]. Around 70% of the gastric cancer patients are considered to be incurable at

*Correspondence to*: Shao-Xuan Wang, MM. Department of Gastroenterology, Shandong Jining No.1 People's Hospital, Shandong, 272000, China. Tel: +86 537 2253423, E-mail: shaowang120@gmail.com Received: 30/05/2015; Accepted: 14/06/2015 the time of diagnosis due to the already advanced stage and tumor dissemination to other parts of the body. The recurrence rate is also very high in patients with resectable disease, finally leading the majority of them to develop advanced cancer [3]. The vulnerability of the population to gastric cancer relies on several factors comprising of lifestyle, age, environmental and genetic factors. For instance, vitamin C, carotenoids and green tea have been reported to have preventive effects in gastric cancer [4,5].

Surgical resection of operable tumors in combination with local radiotherapy and chemotherapy with conventional anticancer drugs is the standard treatment for gastric cancer patients [6]. Chemotherapy has been thoroughly examined in gastric cancer cases. Numerous drugs have displayed effective anticancer activity; nevertheless, single-drug chemotherapy has been ineffective in improving survival rates. Numerous combination regimens have been recognized with potent efficacy in both locally advanced and metastatic disease. These combinations include epirubicin plus cisplatin plus 5-FU (ECF), etoposide plus leucovorin plus 5-FU (ELF), 5-FU plus high dose methotrexate plus doxorubicin (FAMTX), etoposide 📶 doxorubicin plus cisplatin (EAP) etc [7]. Althoug the efficacy of these combination treatments ar promising, the side effects are enormous and may include anemia, neurotoxicity, and nephrot ty. These side effects result from the non-specific toxicity of these drugs which also target normal cells besides cancer cells. This non-specific toxicity necessitates the development of novel therapeutic agents to treat this deadly cancer [8,9]. Various published reports claim that many natural compounds can be combined with known anticancer agents, enhancing their anticancer activity [10,11], and decreasing side effects since antitumor efficiency can be achieved with lower doses of anticancer drug

Keeping this in view, our current study focused on the enhancement of the anticancer activity of 5-FU by combining it with bornyl acetate - a monoterpenoid natural product present in many plant species. The mode of action of the activity enhancement was also demonstrated by studying the effect on cell apoptosis, cell cycle arrest, DNA damage and caspase protein expressions.

### Methods

#### Chemicals and other reagents

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetra-

zolium bromide], DMSO, and PI were purchased from Sigma (St. Louis, MO, USA). RPMI-1640, penicillin streptomycin and other cell culture supplies were from Gibco BRL (Grand Island, NY, USA). Acridine orange (AO) and Hoechst 33342 were obtained from Beyotime, China. Annexin V-FITC-Propidium Iodide Apoptosis Detection Kit was purchased from Beyotime Institute of Biotechnology, Shanghai, China. The cell-permeable pan-caspase inhibitor, the caspase-3 specific inhibitor and the caspase-8 specific inhibitor were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. All other chemicals and solvents used were of the highest purity grade.

#### Cell line

Human gastric cancer cell line (SGC-7901) was obtained from the China Center for Type Culture Collection (Wuhan, China). These cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were cultured in a CO<sub>2</sub> incubator with an internal atmosphere of 95% air and 5% CO<sub>2</sub> and the cell lines were maintained at 37°C.

### Cell viability testing by MTT assay

Briefly, SGC-7901 cells were seeded in a 96 multi-well plate (5x10<sup>2</sup> cells/100 µl), and incubated at 37°C. After 24 hrs, the cells were treated with 0, 1.5, 3, 6, 12, 24, 48 and 96 µM bornyl acetate, 1.5 µM 5-FU or their combination (48 µM bornyl acetate + 1.5 µM 5-FU) for 48 hrs. After incubation, MTT (2.0 mg/ml) was added to each well, and the plates were incubated in the dark at 37°C for 2 hrs. The medium was removed, formazan crystals were dissolved in DMSO, and the optical density (OD) was measured at 570 nm using an ELISA plate reader. The cell viability index was calculated according to the formula: (experimental OD value/control OD value) × 100%.

Cytotoxicity was expressed as the concentration of bornyl acetate inhibiting cell growth by 50% (IC<sub>50</sub> value).

#### Assay for colony formation

In brief, 5.0 mL RPMI 1640 medium with 0.8% agar were added into each well of the 6-well plate. After the medium became solid, 10000 cells were suspended in RPMI 1640 medium with 0.5% agar and seeded on the top of the basal gel. Cells were then treated with 48  $\mu$ M bornyl acetate, 1.5  $\mu$ M 5-FU or their combination for 48 hrs, and incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> for 1 week. Subsequently, cells were fixed with 4% formaldehyde and stained with 0.5 mL 0.2% crystal violet for 2 hrs. The cells were then rinsed with PBS and the number of colonies were finally measured using a microscope.

Phase contrast microscopic evaluation of the cell death in

#### SGC-7901 cells

Briefly, SGC-7901 human gastric cancer cells were plated at a density of  $2x10^5$  cells/ml and then cultured for 24 hrs to allow attachment of the cells to the surface of 6-well plates. Afterwards, the cells were treated with 48  $\mu$ M bornyl acetate, 1.5  $\mu$ M 5-FU or their combination for 48 hrs. Following drug treatment, culture plates were observed with a phase contrast microscope (Olympus, Center Valley, PA, USA) and images were captured.

## Fluorescence microscopic evaluation of cell death using Hoechst33342 and acridine orange staining

SGC-7901 human gastric cancer cells which were grown on coverslips in 12-well plates were subjected to treatment with 48  $\mu$ M bornyl acetate, 1.5  $\mu$ M 5-FU or their combination for 48 hrs. Then the cells were washed twice with PBS and stained with Hoechst 33342 and acridine orange (Hoechst Staining Kit, Beyotime, China) for 1 h at room temperature. Fluorescence microscopy was used to detect cell shape captured from various random visual fields. The ratio of apoptotic cells to total cell number was calculated. Fluorescence signal was detected and recorded using a fluorescence microscope (Nikon, TS-100-F, New York, USA) equipped with a digital camera (Nikon, DS-5Mc, New York, USA).

### DNA fragmentation analysis by gel electrophore

Following treatment of SGC-7901 cells with 48  $\mu$ M bornyl acetate, 1.5  $\mu$ M 5-FU or their combination for 48 hrs, both adherent and floating cells were collected and washed with PBS. Pellets were then lysed with DNA lysis buffer (25 mM EDTA, 75 mM Tris, Ph

7.2, 0.5% SDS) at room temperature for 20 min. After centrifugation for 10 min at  $20,000 \times g$ , the supernatants were collected and treated with RNase A (final concentration, 500 µg/ml) for 30 min at 37°C, followed by digestion with proteinase K (final concentration 500 µg/ml) for 1.5 hrs at 50°C. The DNA was extracted using the phenol/chloroform/isoamylol (25:24:1), precipitated with ethanol, dissolved in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and subjected to 2% agarose gel electrophoresis for DNA fragmentation analysis.

## Flow cytometric evaluation of the cell cycle phase distribution

Briefly, SGC-7901 cancer cells  $(2 \times 10^5)$  were seeded into each well of 6-well plates and incubated for 24 hrs. The cells were treated with 48 µM bornyl acetate, 1.5 µM 5-FU or their combination for 48 hrs. Untreated cells (control) were also incorporated. After incubation for 24 hrs, the cells were harvested and fixed with ice-cold 70% ethanol (5 mL) at  $-20^{\circ}$ C for 2 hrs. Prior to analysis, the cells were washed with cold PBS and re-suspended in 500 µl of PBS, 10 µl PI and 10 µl RNase A The DNA contents were recorded by a flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest software, measuring the fluorescence emission at 530 nm and 575 nm using 488 nm excitation.

## Apoptotic/necrotic cell death evaluation by Annexin V-FITC/propidium iodide double-staining assay

SGC-7901 cancer cells were treated with 48  $\mu M$  bornyl acetate, 1.5  $\mu M$  5-FU or their combination for 48 hrs. The cells were trypsinized, rinsed twice with PBS, and resuspended in 1× binding buffer. The cells were



**Figure 1.** Effect of bornyl acetate on the proliferation of SGC-7901 cancer cells at increasing concentrations. \*p<0.05 vs control group. \*\*p<0.01 vs control group.

labeled with 5 µl of FITC-conjugated annexin V and 10 µl of PI. The cells were incubated for 30 min in the dark at 37°C and then 500 µl of binding buffer was added and the samples were immediately analyzed with a flow cytometer (Becton Dickinson, San Jose, CA). The annexin V-FITC-/PI- cell population was considered as normal, while the annexin V-FITC+/PI- and Annexin V-FITC+/PI+ cell populations were considered as indicators of early and late apoptotic cells, respectively.

#### Statistics

Statistical analysis of the data was carried out using Prism 5.0 (Graph Pad Software, CA, USA). All the data were expressed as mean ± standard error of mean (S.E.M.). The significance of difference between groups was estimated by one-way analysis of variance (ANO-VA) followed by Dunnett's post-hoc test. A p value less than 0.05 indicated statistical significance.

### Results

# *Combined effect of bornyl acetate and 5-FU on SGC-7901 human gastric cancer cell viability*

With the aim of examining the combined effect of bornyl acetate and 5-FU on the SGC-7901 cell proliferation, the cells were treated with various concentrations of bornyl acetate and 5-FU for 48 hrs separately. Then the cell viability was evaluated by MTT assay. We found that both bornyl acetate and 5-FU induced a growth inhibitory effect on these cells in a dose dependent manner (Figure 1). Based on these findings, we were able to select a moderate dose (48 µM bornyl ac-

etate+1.5  $\mu$ M 5-FU) for combination treatment. The combination experiment revealed that, as compared to separate treatment by bornyl acetate or 5-FU, combination of these two inhibited cancer cell growth much more effectively (Figure 2) indicating that bornyl acetate potentiates the antitumor efficacy of 5-FU.

Further, we evaluated the anti-clonogenic effects of the combination treatment which indicated that bornyl acetate and 5-FU combination decreased the number of cancer cell colonies in comparison to the individual treatment. When taken together, these results indicate that the combination of these two compounds inhibits both anchorage-dependent (antiproliferative) as well as anchorage-independent (colony formation) growth of SGC-7901 cancer cells (Figure 3).

Combined effect of bornyl acetate and 5-FU on SGC-7901 human gastric cancer cell death via apoptosis

The cell death induced by bornyl acetate, 5.FU or their combination was evaluated by phase contrast and fluorescence microscopy. Following incubation with 48  $\mu$ M bornyl acetate, 1.5  $\mu$ M 5.FU or their combination for 48 hrs, the most obvious apoptotic changes were observed in the treated cells. As shown by phase contrast microscopy (Figure 4), the untreated control cells were morphologically normal. Reduction in the cell population and change in cellular morphology were observed with the combination treatment with bornyl acetate and 5-FU.

To determine whether the features of cell



**Figure 2.** Combination effect of bornyl acetate (48  $\mu$ M) and 5-FU (1.5  $\mu$ M) on the proliferation of SGC-7901 cells. \*p<0.05 vs control group. \*\*p<0.01 vs control group.



**Figure 3.** Bornyl acetate and 5-FU combination inhibited the colony formation of SGC-7901 cancer cells. **A** represents control group, **B** shows 48  $\mu$ M cose of bornyl acetate, **C** shows 1.5  $\mu$ M of 5-FU while **D** shows combination of bornyl acetate (48  $\mu$ M) + 5-FU (1.5  $\mu$ M).

death induced by the combination of bornyl acetate and 5-FU were from apoptosis or necrosis, the patterns of morphological changes were investi-gated. Fluorescence microscopy using Hoechst 33342 and Acridine orange staining revealed morphological features including chromatin condensation, fragmented nuclei and nuclear shrinkage which increased at the onset of combination treatment (Figure 5 A-D and Figure 6 A-D) after 48 hrs. Figure 5 shows morphological changes in SGC-7901 cells that had been treated with 48  $\mu$ M bornyl acetate, 1.5 µM 5-FU or their combination. The cells developed obvious apoptotic features that included chromatin condensation and nuclear fragmentation (Figure 5 and Figure 6). On the contrary, no such changes were seen in untreated cells. In case of Acridine orange staining, green intact nuclei indicated normal cells, while as orange staining indicated apoptosis. The number of cells with orange staining increased with combi-

nation treatment (Figure 6).

Effect of bornyl acetate and 5-FU combination on DNA fragmentation in SGC-7901 human gastric cancer cells

Besides the morphological changes of apoptosis in bornyl acetate plus 5-FU combination-treated SGC-7901 cells, DNA fragmentation analysis was also examined by observation of the formation of DNA ladder. The cells were treated with bornyl acetate (48  $\mu$ M), 5-FU (1.5  $\mu$ M) or their combination for 48 hrs. As shown in Figure 7 A, DNA ladder seemed to be more obvious with the onset of combination treatment, however, no DNA fragments were observed in the control groups (Figure 7 A). Similarly, DNA fragmentation was also observed at different time intervals using bornyl acetate and 5-FU combination. The results (Figure 7 B) revealed that the combination



**Figure 4.** Bornyl acetate and 5-EU combination induced potent morphological changes (white arrows) in SGC-7901 gastric cancer cells. Cells were untreated (**A**), and treated with bornyl acetate (48  $\mu$ M, **B**), 5-FU (1.5  $\mu$ m, **C**) or their combination bornyl acetate (48  $\mu$ M) + 5 FU (1.5  $\mu$ M, **D**) for 48 hrs.

also induced time-dependent DNA fragmentation.

Combination effect of bornyl acetate and 5-FU on the apoptosis/necrosis induction in SGC-7901 cells

The influence of the combination treatment on apoptosis induction in SGC-7901 cells was additionally verified by Annexin V/PI staining using flow cytometry. SGC-7901 cells were treated with 48  $\mu$ M bornyl acetate, 1.5  $\mu$ M 5-FU or their combination for 48 hrs, and were then examined by flow cytometry to verify apoptosis. As shown in Figure 8 A-D, bornyl acetate and 5-FU alone did induce apoptosis since the fraction of early and late apoptotic cells increased in comparison to the control cells. Compared to individual treatment by bornyl acetate and 5-FU alone, the percentage of apoptotic cells induced by their combination was considerably much higher (Figure 8 D). The percentage of apoptotic cells increased in the following manner: untreated control (apoptotic cells were 4.4%), bornyl acetate (48  $\mu$ M) (apoptotic cells were 15.4%), 5-FU (1.5  $\mu$ M) (apoptotic cells were 39%) and bornyl acetate (48  $\mu$ M) +5-FU (1.5  $\mu$ M) (apoptotic cells were 55.9%). This experiment lends support to the claim that bornyl acetate potentiates 5-FU in inducing apoptosis in gastric cancer cells by increasing the number of early and late apoptotic cells.

# Combination of bornyl acetate and 5-FU induced a potent G2/M cell cycle arrest in SGC-7901 cancer cells

In order to establish whether the combination of bornyl acetate with 5-FU could potentiate its efficacy in disturbing cell cycle phase distribution, SGC-7901 cells were treated with 48  $\mu$ M bornyl acetate, 1.5  $\mu$ M 5-FU or their combination for 48 hrs, and then flow cytometry was performed to examine the effect on cell cycle phase distribution.



**Figure 5.** Morphological features of apoptosis including chromatin condensation induced by the combination of bornyl acetate (48  $\mu$ M, **B**), 5-FU (1.5  $\mu$ M, **C**) or their combination bornyl acetate (48  $\mu$ M) + 5-FU (1.5  $\mu$ M) (**D**) for 48 hrs. **A** represents the untreated control group. White arrows represent morphological changes in the gastric cancer cells. Combination treatment achieved the maximal damage. Hoechst 33342 was used as a staining agent and fluorescence microscopy was used to capture the images.



**Figure 6.** Morphological features of apoptosis induced by the combination of bornyl acetate (48  $\mu$ M, **B**), 5-FU (1.5  $\mu$ m, **C**) or their combination bornyl acetate (48  $\mu$ M) + 5-FU (1.5  $\mu$ M) (**D**) for 48 hrs. **A** represents the untreated control group. White arrows show orange fluorescence of apoptotic cells. Combination treatment shows the maximal damage. Acridine orange was used as a staining agent and fluorescence microscopy was used to capture the images (400×). Green intact nuclei indicate normal cells, while orange staining indicates apoptosis.



**Figure 7.** Analysis of bornyl acetate and 5-FU combination-induced DNA fragmentation in SGC-7901 cancer cells. The cells were treated with bornyl acetate (48  $\mu$ M), 5-FU (1.5  $\mu$ M) or their combination bornyl acetate (48  $\mu$ M) + 5-FU (1.5  $\mu$ M), for 48 hrs. DNA fragmentation was analyzed by agarose gel electrophoresis and staining with ethidium bromide, as described in the Methods section. **A** shows DNA fragmentation induced by bornyl acetate (B.A.) and 5-FU and their combination, while **B** represents DNA fragmentation induced by the combination of B.A and 5-FU only at different time intervals.

As shown in Figure 9 A-D, the percentage of cells in G2/M phase of the cell cycle witnessed a sharp increase as the cells were exposed to the combination treatment. Figure 9 A shows the untreated (control) cells with very low G2/M percentage. This experiment further demonstrated that bornyl acetate forms a suitable combination with 5-FU in killing cancer cells through inducing G2/M phase cell cycle arrest.

### Discussion

The current anticancer treatments are based mostly on drug combinations. Chemotherapy for treating gastric cancer has no decisively established standard of care. Unfortunately, gastric cancer has not been principally susceptible to chemotherapeutic drugs currently in use. Therefore, chemotherapy has been used to reduce the tumor size, relieve symptoms of the disease and possibly prolong survival. The main drugs used for gastric cancer include 5-FU, capecitabine, carmustine, doxorubicin, cisplatin or combinations of these drugs [12]. However, their use is restricted due to serious side effects including anemia, nephrotoxicity, and neurotoxicity, cardiac arrest, myelosuppression, excessive nausea/vomiting etc. This is further complicated by the drug resistance problem acquired by the cancer cells [13]. To address these problems, investigations have been done on identifying novel plant agents that can be combined with 5-FU or other known drugs to increase the therapeutic efficacy and reduce side effects.

Taking this into consideration, our study focused on bornyl acetate - an essential oil constituent present in many plants. The aim was to demonstrate whether the combinations of bornyl acetate and 5-FU could result in more significant anticancer effect than either of the two drugs used alone. Our MTT results showed that, as compared to separate treatment by bornyl acetate or 5-FU, the combination of these two inhibited cancer cell growth much more efficiently, indicating that bornyl acetate potentiates the antitumor efficacy of 5-FU. We also evaluated the anti-clonogenic effects of the combination treatment which indicated that bornyl acetate and 5-FU combination decreased the number of gastric cancer cell colonies in comparison to the individual treatment. Phase contrast microscopy revealed reduction in the cell population and change in cellular mor-



**Figure 8.** Bornyl acetate and 5-FU combination-induced apoptosis of SCC 7901 cells analyzed by FACS and stained with annexin V-FITC/PI. Cells were treated with either 48 µM bornyl acetate (B.A, **B**), 1.5 µM 5-FU (**C**), or their combinations (B.A+5-FU, **D**) for 48 hrs, while (**A**) served as control. Data summary and analysis of the proportion of SGC-7901 cells in different periods was according to the results of flow cytometric analysis.



**Figure 9.** Effect of bornyl acetate (48  $\mu$ M, **B**), 5-FU (1.5  $\mu$ M, **C**), and their combinations (**D**) on cell cycle phase distribution in SGC-7901 cells using FACS analyzer. SGC-7901 cells were stained with propidium iodide after 48 hrs exposure to control (**A**), 48  $\mu$ M bornyl acetate, 1.5  $\mu$ M 5-FU (**C**), or their combination (**D**). Data is representative of three independent experiments. G2/M cell population witnessed a sharp increase with the combination treatment.

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phology with the combination treatment using bornyl acetate and 5-FU. Fluorescence microscopy showed that the gastric cancer cells developed evident apoptotic features that included chromatin condensation and nuclear fragmentation after combination treatment with 5-FU and bornyl acetate. DNA fragmentation assay using gel electrophoresis revealed that DNA fragments became obvious with the onset of combination treatment, however, no DNA fragments were observed in the control groups. Similarly, DNA fragmentation was also observed at different time intervals using bornyl acetate and 5-FU combination. The results revealed that the combination also induced time-dependent DNA fragmentation. Compared to treatment by bornyl acetate and 5-FU alone, the percentage of apoptotic cells induced by their combination was considerably much higher. Further mechanistic details using cell cycle analysis revealed that the combination of 5-FU and bornyl acetate induced G2/M cell cycle arrest in these gastric cancer cells.

In conclusion, bornyl acetate potentiates the anticancer activity of 5-FU in SGC-7901 human gastric cancer cells by inducing apoptosis, DNA fragmentation as well as G2/M cell cycle arrest.



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