

## Anticancer Effect of Emodin Combined with 5-fluorouracil on Human Gastric Carcinoma Cell Line MKN45 and its Molecular Mechanism

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### ABSTRACT

**AIM:** The purpose of this study was to investigate the anticancer effect induced by and 5-fluorouracil (5-Fu) on human gastric carcinoma cell line MKN45, and to discuss its molecular mechanism.

**METHODS:** Firstly, MTT assay was used to assess the growth inhibition effect of emodin and 5-Fu. Secondly, Flow cytometry was used to monitor the changes of cell cycle distribution and decipher the suppressive mechanisms of emodin on MKN45 cells. Thirdly, Acridine Orange staining was used to observe the nuclear morphological changes that occurred during emodin treatment. Finally, immunoblot analysis of proteins p53 and p21<sup>waf1</sup> derived from emodin-treated MKN45 cells were analyzed to evaluate the mechanism of cell apoptosis induced by emodin.

**RESULTS:** It showed that emodin induced cell death in a dose- and time-dependent manner and 10, 20 µg/mL of emodin combined with 10 mg/L 5-Fu significantly inhibited cell growth. Moreover, the G<sub>2</sub>/M phase blocking could be detected by flow cytometry. Apoptosis which correlated with concentration of emodin could also be observed by Acridine Orange staining. In addition, when exposed to emodin for 48 hours, a dose-dependent increase of proteins p53 and p21<sup>waf1</sup> was observed, which indicated the potential mechanism of apoptosis induced by emodin. These studies suggested that emodin was a suitable and novel chemotherapeutic drug candidate for the treatment of human gastric carcinoma.

**CONCLUSION:** These studies suggested that emodin was a suitable and novel chemotherapeutic drug candidate for the treatment of human gastric carcinoma.

### INTRODUCTION

Gastric cancer (GC) is one of the most prevalent tumors, it remains the second leading cause of cancer death worldwide, especially in Asia<sup>[1]</sup>. However, conventional strategies for treatment of gastric cancer are not yet satisfactory. Therefore, the development of new drugs for the treatment of gastric cancer is of great importance.

Rhubarb (Rheum) species have a long history as medicinal plants in traditional Chinese medicine. Rhei Rhizoma (Dahuang) is a Chinese herbal medication that has traditionally been prescribed for its purported purgative and anti-inflammatory properties<sup>[2]</sup>. According to the Chinese Pharmacopoeia, Rhei Rhizoma is derived from one of three Rheum species, Rheum palmatum, R. tanguticum, and R. officinale. The main active ingredients of the Rheum species are a series of anthraquinones, dianthrone, glycosides and tannins. The anthraquinone derivatives include emodin, rhein, chrysophanol, physcion, alizarin, citreorosein, and aloe-emodin. Some studies have indicated that emodin has a number of biological properties, including antiviral, antimicrobial, and hepatoprotective activities. Recently emodin has also been reported to exhibit anticancer activity<sup>[3]</sup> on human breast cancer cells which highly expressed HER2/neu<sup>[2,4,5]</sup>, lung adenocarcinoma cells A549, bladder cancer cells<sup>[6,7]</sup>, liver cancer cells HepG2, pancreatic cancer<sup>[8]</sup>, non-small cell lung cancer (NSCLC) cells H460D<sup>[9]</sup>, human cervical cancer cell lines<sup>[10]</sup>, and chronic myelogenous leukemia (CML) cells K562<sup>[11-14]</sup>.

5-Fluorouracil (5-Fu) is one of the most common used chemotherapeutic drugs on gastric cancer. It can change into 5-Fu deoxynucleotide, which can inhibit thymidylate synthase, block the methylation of deoxyguanylic acid (dGMP), and influence

the synthesis of DNA. Moreover, 5-Fu can also change into 5-Fu nucleoside, which can mix into RNA as the form of false metabolite, then influence the synthesis of proteins<sup>[15]</sup>. Although 5-Fu has cytopathic effect to cancer cells, it can also kill normal cells. Therefore, tasks of looking for new efficient and harmless drugs or combined therapeutic strategy are critical. It was reported that some of conjugates such as 5-Fu and emodin exhibited better or comparable *in vitro* antitumor activity and low toxicity in the normal cell<sup>[16]</sup>.

In this study, we explored the effect of emodin on gastric cancer cells MKN45, and we also combined emodin with 5-Fu when doing the research. The aim was to investigate the potential anticancer effects of emodin and its auxiliary function to 5-Fu, and to discuss the underlying mechanisms.

## METHODS

### Emodin and 5-fluorouracil

Emodin [1, 3, 8-trihydroxy-6-(hydroxymethyl)-anthraquinone, purity  $\geq 97\%$ ] was provided by Institute of pharmacology in Lanzhou University. It was dissolved in 1% dimethylsulfoxide (DMSO) to a concentration of 7.4 mmol/L and stored at  $-20^{\circ}\text{C}$  until used. The concentration of emodin used in this study was 10, 20, 40 and 60  $\mu\text{mol/L}$ . 5-fluorouracil (5-Fu, 0.25g/mL) was purchased from Shanghai Xudong Haipu Pharmaceutical Co.Ltd and the concentration used in this study was 10 mg/L.

### Cells and Cell culture

Human gastric carcinoma cell line MKN45 was obtained from Institute of Cell Biology in Chinese Academy of Sciences (Shanghai). Cells were cultured in plastic flasks or multi-well plates at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  with RPMI-1640 Medium (Gibco, Rockville, USA) containing 10% fetal calfserum (FCS, Gibco, Rockville, USA) and 100 U/mL penicillin (Gibco, Rockville, USA) and 100  $\mu\text{g/mL}$  streptomycin (Gibco, Rockville, USA). Exponentially growing cells were used for experiments.

### MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

Exponentially growing MKN45 cells were digested by 0.25% trypsin for 1-2 min, and washed three times with PBS. RPMI 1640 medium containing 10% newborn bovine serum was added to obtain a cell density of  $5 \times 10^8/\text{L}$ . Final cell suspensions (100  $\mu\text{L}$ ) were placed in 96-well plates in an incubator containing 5%  $\text{CO}_2$ , and incubated at  $37^{\circ}\text{C}$  for 24 h. Then, 100  $\mu\text{L}$  RPMI 1640 medium containing different concentrations of emodin and 5-Fu was added to each plate. Set of three wells were used for each dose and for negative control (0.1% DMSO) in this assay. When cells were treated for 24 h, 48 h and 72 h, 30  $\mu\text{L}$  of MTT solution (2 mg/mL, in phosphate buffered saline, PBS) were added to each well of 96-well plates. After cells were incubated at  $37^{\circ}\text{C}$  for another 4 h, the medium was removed and 150  $\mu\text{L}$  of DMSO was added to each well to resolve the formazan. The microplate was then shaken on a rotary platform for 10 min. The Optical density (OD) values were measured at 570 nm using a Multimode Reader (ELx800, Bio-TEK, USA). Inhibitory rate (IR) was calculated according to the following formula:  $\text{IR}\% = [1 - (\text{mean of treated group}) / (\text{mean of control group})] \times 100\%$ .

### Flow cytometry (FCM) assay

For flow cytometric analysis of cell cycle,  $1 \times 10^6$  MKN45 cells were treated with or without various concentrations of emodin (10, 20, 40 and 60  $\mu\text{g/mL}$ ) or combined with 5-Fu (10 mg/L) for 48 h in an incubator containing 5%  $\text{CO}_2$ . Cells were then digested with 0.25%

trypsin and sedimented by centrifugation at 3000 rpm (937 g) for 5 min at room temperature. After the supernatant was removed, the cell suspensions were fixed with 70% cold ethanol for 24 h. After centrifugation for 5 min at 200 g, the cells were resuspended in 0.5 mL PBS, and then were digested by RnaseA for 30 min at  $37^{\circ}\text{C}$ . The final cell suspensions were stained with 1mL Propidium Iodide (50 mg/mL) for 30 min and were analyzed for cell cycle using Coulter Flow Cytometer (Beckman Coulter, Inc., Miami, FL, USA) at excitation wavelength 488 nm and absorption wavelength 600 nm. The distributions of three phases were estimated according to standard procedures. The percentages of cells in different cell cycle phases ( $G_0/G_1$ , S or  $G_2/M$  phase) were calculated by means of Coulter Epicx XL-MCL DNA Analysis Software. Three wells of a 6-well plate were used for each dose and timed treatment. Three independent experiments were performed in this analysis.

### AO (Acridine Orange) staining

Exponentially growing MKN45 cells were digested by 0.25% trypsin for 1-2 min, and washed three times with PBS. The cell suspension was transferred into 6-well plate which was filled with a Cover Slip in it, and then were treated with various concentrations of emodin (10, 20, 40 and 60  $\mu\text{g/mL}$ ) or combined with 5-Fu (10 mg/L) for 48 h. The Cover Slips were fetched out and fixed with 95% ethanol for 15 min, then 5  $\mu\text{L}$  AO (100 mg/L) were added. These Cover Slips were observed and photographed immediately with a Fluorescence Microscope (Olympus AX 80, Japan).

### Western Blot analysis for proteins p53 and p21<sup>waf1</sup>

After exponentially growing MKN45 cells were treated with various concentrations of emodin (10, 20, 40 and 60  $\mu\text{g/mL}$ ) or combined with 5-Fu (10 mg/L) for 48 h, cell lysate was made by adding RIPA Lysis Buffer (Beyotime, Jiangsu, China) containing 1 mM PMSF (phenylmethyl sulfonyl fluoride). The lysate was sonicated for 30 seconds on ice and then centrifuged at 10000 rpm for 5 min at  $4^{\circ}\text{C}$ . Protein was quantitated by BCA (bicinchoninic acid) method. Total protein (20  $\mu\text{g}$ ) per sample was subjected to electrophoresis on 10% sodium dodecylsulfate-polyacrylamide gel and electrotransferred to nitrocellulose membrane. After blocking with phosphate buffered saline (PBS) containing 5% powdered milk for 2 hours, the membrane was incubated with polyclonal antibody against the primary antibodies: mouse monoclonal anti-p53 and polyclonal anti-p21<sup>waf1</sup> (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), diluted 1:1000, for 2 hours at  $37^{\circ}\text{C}$ . The membrane was washed with PBS for 3 times and 5 min per time, followed by incubation with fluorescent labeled secondary antibodies (goat anti-mouse IgG, Beyotime, Jiangsu, China), diluted 1:2000. After extensive washing the protein was visualized at Fast Chemiluminescence Image System (ImageQuant 350, GE Healthcare, China). The protein of  $\beta$ -actin (Abcam Ltd., Cambridge, UK) was used as an internal control. All experiments were conducted three times, independently.

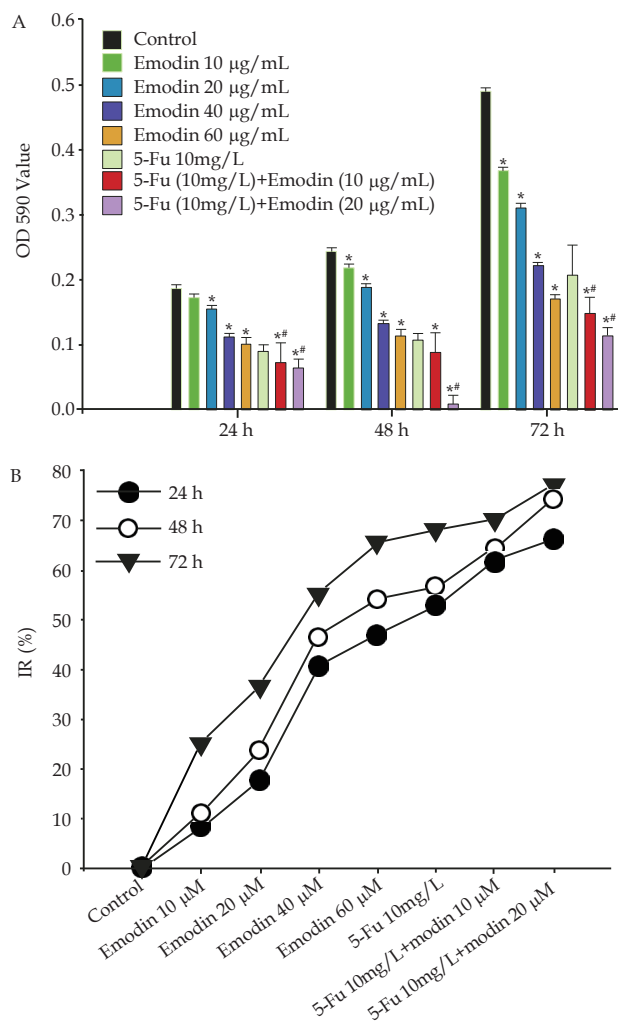
### Statistical analysis

Statistical analysis was performed using SPSS software 10.0 (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was used to make a statistical comparison between groups. The level of significance was set at  $p < 0.05$ .

## RESULTS

### Emodin inhibits cell growth in a dose- and time-dependent manner

To assess the growth inhibition effect of emodin and combined with 5-Fu, the MTT assay of gastric carcinoma cells MKN45 was made

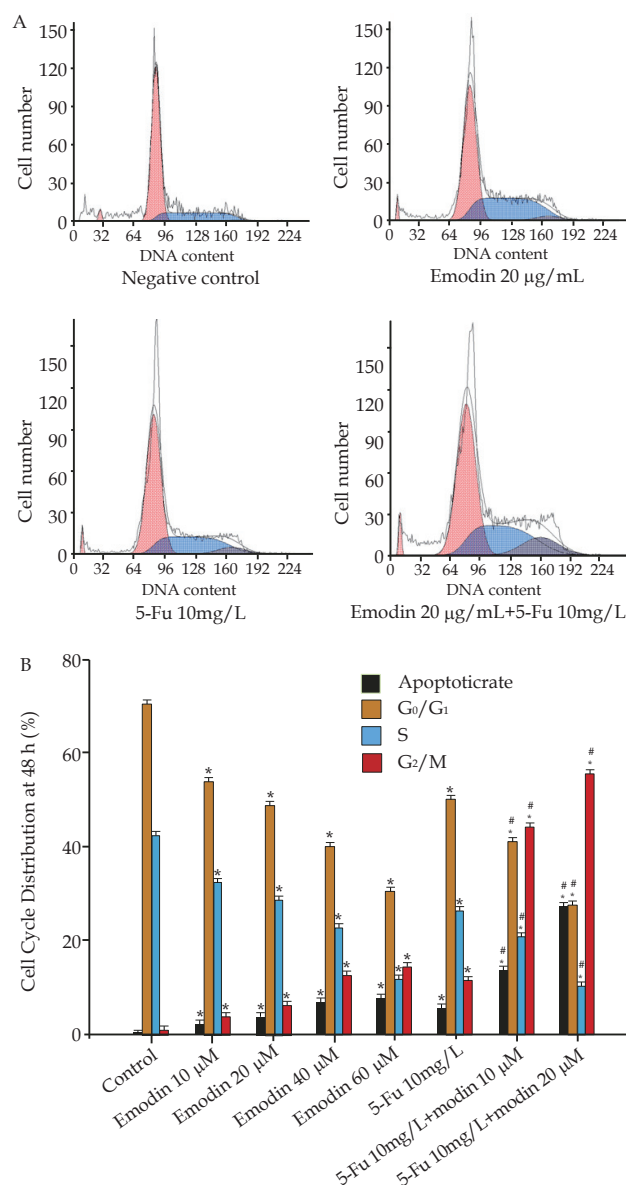


**Figure 1** Growth inhibition effect of emodin on gastric carcinoma cells. MKN45 Cells were treated with 10, 20, 40, 60 µg/mL emodin or combined with 10 mg/L 5-Fu at the indicated times. (A) Optical density values at 570 nm for each group were measured ( $n=5$  for all groups). (B) Inhibitory rates (IR%) of all groups were calculated and shown at 3 different time points. Error bars indicate the mean±SEM. \*  $P<0.05$  comparing with control group. #  $p<0.05$  comparing with 5-Fu group.

according to a standard operating procedure. Exposure to various concentrations of emodin (10, 20, 40 and 60 µg/mL) or combined with 5-Fu (10 mg/L), the MKN45 cells showed a dose- and time-dependent decrease in cell viability in contrast with control group (Figure 1A). The cell inhibition rate (IR %) of groups which treated with emodin (both 10 and 20 µg/mL) combined with 5-Fu were higher and had significant differences compared with control group and 5-Fu group (Figure 1B). When exposed to emodin for 72 h, the  $IC_{50}$  value was nearly 40 µg/mL (Figure 1B).

### Emodin arrests MKN45 cells at G<sub>2</sub>/M phase

In order to decipher the suppressive mechanisms of emodin on MKN45 cells, we monitored the changes of cell cycle distribution using flow cytometry. Compared to the control group, after treatment of emodin for 48 h, an obvious Sub-G<sub>1</sub> peak could be seen in the DNA analysis histogram (Figure 2A). The cell cycle distribution analysis revealed that the number of cells in G<sub>2</sub>/M phrase and the Apoptosis Rate were greatly increased, while cells at S phase were gradually decreased with increasing dose of emodin (10, 20, 40, 60 µg/mL) compared with the control group (Figure 2B). Combining emodin with 5-Fu could also increase the number of cells in G<sub>2</sub>/M phrase and the apoptosis rate, while decrease the number in S phase comparing

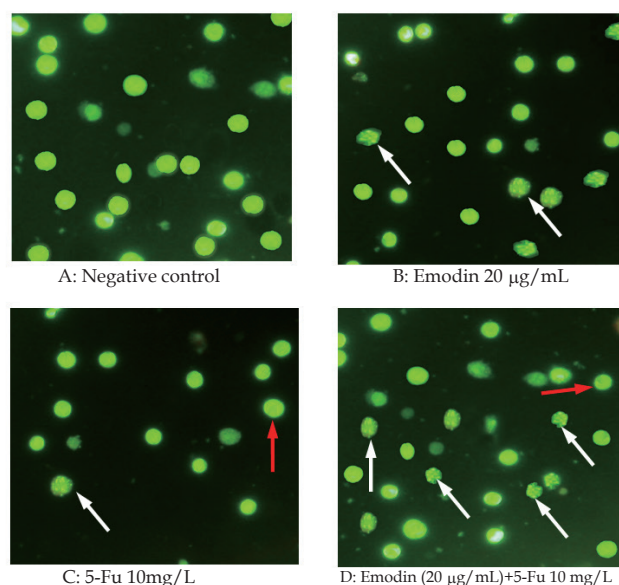


**Figure 2** Cell cycle distribution analysis of emodin on gastric carcinoma cells. MKN45 cells were treated with 10, 20, 40, 60 µg/mL emodin or combined with 10 mg/L 5-Fu for 48 h. (A) Cell cycle distribution by FCM. (B) The statistical analysis for each cycle distribution. Error bars indicate the mean±SEM. \*  $P<0.05$  compared with control group. #  $p<0.05$  compared with 5-Fu group.

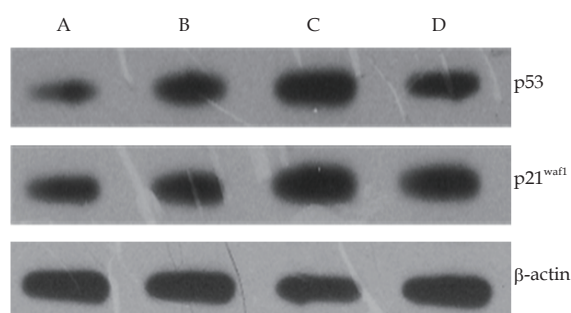
with 5-Fu group (Figure 2B).

### Emodin induces cell death through the apoptotic pathway

To further investigate whether the emodin-mediated cell death in MKN45 cells was due to an apoptotic mechanism, the nuclear morphological changes that occurred during emodin treatment were observed. Treatment of MKN45 cells with various concentration emodin for 48 h resulted in changes in the nuclear morphology as evidenced by the AO staining (Figure 3), a dye which is often used to label DNA in living cells and to observe morphological and nuclear changes. Compared to the negative control group, when cells were treated with emodin, the volume were shrink, the nuclei appeared pyknosis, the chromatin concentrated and gathered at the border of nuclei to form a bulk, ring or crescent, and the apoptotic bodies were observed (figure 3B). Compared to the 5-Fu group, when emodin combined with 5-Fu, cell morphological and nuclear changes were more serious (figure 3D).



**Figure 3** Cell morphological analysis of Emodin on gastric carcinoma cells. After treatment with (A) negative control (B) Emodin 20 µg/mL (C) 5-Fu 10mg/L and (D) Emodin 20µg/mL +5-Fu 10mg/L for 48 h, apoptotic cells were detected by AO staining and examined by fluorescence microscopy. The representative images of three independent experiments are shown. Arrows in white indicated the fragmented nuclei; Arrows in red indicated the gathering of nuclei chromatin and the formation of apoptotic bodies. Magnification:×200.



**Figure 4** Western blot analysis of proteins p53 and p21<sup>waf1</sup>. Gastric carcinoma cells MKN45 were treated with negative control (A), Emodin 20 µg/mL (B), 5-Fu 10 mg/L (C) and Emodin 20 µg/mL +5-Fu 10 mg/L (D) for 48 h, proteins p53 and p21<sup>waf1</sup> were detected in triplicates, and β-actin was control.

### Emodin induces cell apoptosis associated with the increase of proteins p53 and p21<sup>waf1</sup>

In order to evaluate the mechanism of cell apoptosis induced by emodin, immunoblot analysis of proteins p53 and p21<sup>waf1</sup> derived from emodin-treated MKN45 cells were analyzed. In contrast to the control group, after MKN45 cells were treated with emodin for 24 hours, the expression of p53 and p21<sup>waf1</sup> did not have significant changes (data not shown). However, when treated with emodin for 48 hours, a dose-dependent increase of p53 and p21<sup>waf1</sup> was observed (Figure4). When cells were treated with emodin combined with 5-Fu, the expression of p53 and p21<sup>waf1</sup> also increased compared with group treated with emodin alone (Figure 4).

## DISCUSSION

In the present study, we demonstrated that emodin has an anticancer activity on MKN45 gastric carcinoma cells in a time- and dose-dependent patterns by MTT assay. More importantly, we found that when cells were treated with emodin and 5-Fu, the growth inhibitory effect was much more obvious compared to each emodin group and

5-Fu group. For example, when MKN45 cells were treated with 20 µg/mL emodin combined with 10 mg/L 5-Fu for 72 h, the inhibition rate was twice as much as that of 20 µg/mL emodin group, and more than doubled than that of 5-Fu group. This indicated that emodin had not only cytopathic effect of gastric cancer cells MKN45, but also assisted with the anti-tumor function of 5-Fu.

It is well known that one of the mechanisms of chemotherapeutic drugs is the interference on cell cycle. In order to decipher the suppressive mechanisms of emodin on MKN45 cells, the changes of cell cycle distribution were evaluated by FCM. The results indicated that treatment of emodin resulted in a dose-dependent increase in the distribution of cells at G<sub>2</sub>/M phase (Figure 2). Our data were consistent with the previous studies<sup>[17-19]</sup> and suggested that emodin can affect the cell cycle distribution.

In our study, it was observed by AO staining analysis that the cytotoxic mechanism involves the induction of apoptosis. AO can penetrate the intact cell membrane and embed into cell nucleus DNA, which then emits out bright green fluorescence. Under the fluorescence microscope, two typical cell morphologies can be observed: viable cell (VN) showed the green normal nucleus chromatin and the viable apoptotic cells (VA) showed the pyknosis or ball green chromatin. As shown in figure 3, when gastric carcinoma cells MKN45 were treated with emodin, the VA cells were detected, which suggested that the cytotoxic effects of emodin might be assisted with apoptotic process<sup>[20]</sup>.

We further investigated the underlying molecular mechanism of emodin-induced apoptosis and the result demonstrated that it was related with the increase of proteins p53 and p21<sup>waf1</sup>. P53 is one of the essential proteins for apoptosis starting, for it control G<sub>2</sub> checkpoint through cyclinB1. P21 plays important roles in growth inhibition caused by DNA damage, and in the transition between G<sub>2</sub> and M phase. P53 and p21 function together to inhibit the growth of cells and arrest cells in G<sub>2</sub>/M phase. Our data in figure 4 were consistent with the previous research and suggested that emodin-induced MKN45 cells growth inhibition and apoptosis might be related with the increase expression of proteins p53 and p21.

In conclusion, we used human gastric carcinoma cell lines MKN45 to study the anticancer effect of the natural product, emodin, and demonstrated that emodin exhibited an anticancer effect against gastric carcinoma. Its unique cytotoxicity profile together with its promising auxiliary anticancer function for 5-Fu strongly suggested that emodin could be used as a chemotherapeutic drug candidate for the treatment of gastric carcinoma and most likely also other types of human carcinoma. Future studies are to focus on a better understanding of the *in vivo* antitumor function of emodin, the exact mechanism, the mode of action and the cause-effect relationship in order to prepare for the first clinical studies and trials that will evaluate the effect of emodin in cancer patients.

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