

The combination of crocin with cisplatin suppresses growth of gastric carcinoma cell line BGC-823 and promotes cell apoptosis

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Abstract: This study aimed to investigate the efficacy of crocin alone and in combination with cisplatin in the therapy of gastric carcinoma cells. In this study, human gastric carcinoma cell line BGC-823 was purchased and maintained in standard condition. Crocin, cisplatin and crocin plus cisplatin diluted to different concentrations were added into medium, respectively. MTT assay and flow cytometry were performed to test the anti-proliferation effects and apoptosis rates of cells, respectively. In addition, quantitative RT-PCR was used to detect the mRNA expression of apoptosis-related genes, such as *p53*, *Bax* and *Bcl-2*. After treated with different concentrations of crocin, the inhibition ratio and apoptosis rate of BGC-823 cells were not significantly changed. However, the tumor cell inhibition ratio and apoptosis rate in crocin plus cisplatin group were significantly higher than that in cisplatin, crocin and control group ($p < 0.05$). The treatment of crocin plus cisplatin significantly increased the expression of *p53* and *Bax* ($p < 0.05$), and significantly decreased the *Bcl-2* expression ($p < 0.05$). Collectively, our data demonstrated for the first time that crocin plus cisplatin may be used as a new anticancer drug for the treatment of gastric cancer.

Keywords: Gastric cancer, crocin, cisplatin, apoptosis, cell proliferation.

INTRODUCTION

Gastric cancer is one of the most common cancer, and most patients with gastric cancers are diagnosed in advanced stages (Guggenheim and Shah, 2013). It is characterized by marked differences in incidence rates across regions and cultures, and most gastric cancers occur in developing countries, especially in Asian countries (Liu *et al.*, 2014). Cisplatin has been used to treat malignancies such as breast cancer, ovarian cancer, esophageal and gastric cancer (Armstrong *et al.*, 2006; Tepper *et al.*, 2008; Silver *et al.*, 2010; Higuchi *et al.*, 2014). The combination of cisplatin and the 5-fluorouracil (5-FU) related drug S-1 has been established as the first-line chemotherapy for disease in advanced stage in Japan (Koizumi *et al.*, 2008; Sun *et al.*, 2014). However, acquired or intrinsic resistance to cisplatin and the side-effect caused by cisplatin restrict the clinical use of cisplatin to gastric cancer, and it is essential to identify new alternative chemotherapeutic agents that can be used to treat gastric cancer.

Saffron (*Crocus sativus*) is a perennial bulbous plant of the iris family (Iridaceae) (Abdullaev and Espinosa-Aguirre, 2004) and used as anodyne, antidepressant, expectorant, sedative, etc in traditional Chinese medicine. Modern pharmacological studies have demonstrated that saffron possesses anti-tumor activity by inhibiting the growth of tumors (Abdullaev, 1993; Nair *et al.*, 1995). As the pharmacologically active ingredients of saffron

extract, crocin can be used as a pharmacological agent for several diseases. It has been reported that crocin is an antioxidant that inhibits the formation of peroxidized lipids and partly restores SOD activity (Ochiai *et al.*, 2004). Crocin also appears to exhibit hypolipidemic capacity and anticarcinogenic activity (Konoshima and Takasaki, 2003; Sheng *et al.*, 2006). Several studies have documented that crocin inhibits the growth of malignant cancer cells, such as oral squamous cell carcinoma cells (Sun *et al.*, 2011), hepatocarcinoma cells (Noureini and Wink, 2012) and pancreatic cancer cells (Bakshi *et al.*, 2010). However, the effects of crocin on gastric cancer have not been studied before.

In order to explore the role of crocin in the therapy of gastric cancer, the gastric carcinoma cells were treated with crocin, cisplatin and cisplatin plus crocin respectively. Then the inhibition rate of tumor cell growth was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and the cell apoptosis rate was analyzed by flow cytometry. In addition, the detection of apoptosis-related gene expression was used to test the apoptosis of cancer cells as well.

MATERIALS AND METHODS

Cell lines and culture conditions

The human gastric carcinoma cell line BGC-823 was provided by the Cell Bank of Shanghai Institute of Cell

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Biology, Chinese Academy of Sciences. The cells were maintained in RPMI-1640 medium (Hyclone, Logan, UT, USA) with 10% fetal bovine serum (FBS) and 100mg/mL penicillin-streptomycin (Invitrogen/ Gibco, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO₂. The culture medium was renewed every 2-3 days. Cisplatin and crocin were obtained from Sigma (St. Louis, MO, USA). To explore the effect of crocin or cisplatin on gastric carcinoma cells (BGC-823), the cells were treated with different concentrations of crocin and cisplatin, respectively. For combination studies, crocin (Z1: 8mg/mL, Z2: 16mg/mL) plus cisplatin (C1: 0.8µg/mL, C2: 1.6µg/mL) was added to culture medium after the cells attached.

MTT assay

The crocin- or cisplatin-induced inhibition of cell proliferation was measured by the MTT assay. Cells at a density of 6×10⁶ cells per well were seeded in 96-well plates and incubated at 37°C in humidified 5% CO₂ for 24 h. Diluted crocin and cisplatin were added to get the final concentrations. Cells were then incubated for 48h and the MTT assay was carried out according to the instruction from manufacturer. Absorbance values at 490nm were determined on a micro plate spectrophotometer (Bio-Rad, Hercules, CA, USA). Each assay was performed in triplicate. The half maximal inhibitory concentration (IC₅₀) values were calculated.

Flow cytometry for apoptosis rate analysis

The BGC-823 cells at 1.5×10⁵ cells/mL were seeded in 6-well plates and cultured in RPMI-1640 medium containing 10% FBS until the cells attached. The cells were then treated with crocin, cisplatin and crocin plus cisplatin for 48h. Cells were harvested using 0.25% trypsin without EDTA. DNA contents of the samples were analyzed by using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA), and all assays were repeated 3 times.

RNA isolation and quantitative RT-PCR

The cells were harvested after treated with crocin, cisplatin and crocin plus cisplatin for 48h. Total RNA was extracted from BGC-823 cells using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. NP-1000 Spectrometer (Thermo Scientific Hudson, NH, USA) was used to measure integrity and concentration of RNA. Then the first-strand cDNA was synthesized and quantification of mRNA expression levels was determined by using Ssofast EvaGreen Supermix Kit (BIO-RAD) and ABI 7500 system (Applied Biosystems, Carlsbad, Calif., USA). The primers of apoptosis-related genes *p53*, *Bax*, *Bcl-2* and internal control gene β -*actin* were synthesized by Shanghai Sangon Biologic Engineering Technology and Services Co., Ltd. (Shanghai, China), and the sequences of them were shown in table 1. The reaction conditions were:

95°C for 5min; 95°C for 20 s; 55°C for 30 s; 40 cycles. All reactions were run in triplicate. The relative mRNA expression level was calculated by the 2^{- $\Delta\Delta$ Ct} method.

STATISTICAL ANALYSIS

All data are presented as mean \pm standard deviations (SD). Comparison among groups was performed by *t* test and one-way analysis of variance (ANOVA). Pearson correlation analysis was used for the comparison of two variables. SPSS version 17.0 (Chicago, IL, USA) was used for all the statistical analysis, and *p*<0.05 was regarded as significant.

RESULTS

Crocin promoted cisplatin-induced inhibition of BGC-823 cell growth

According to the IC₅₀ curve, the inhibition of gastric carcinoma cell growth was enhanced with the increasing concentration of cisplatin and crocin, and this action was presented in a dose-dependent manner (fig. 1). However, the inhibition effects of crocin on BGC-823 cells were not obvious comparing with cisplatin (fig. 1).

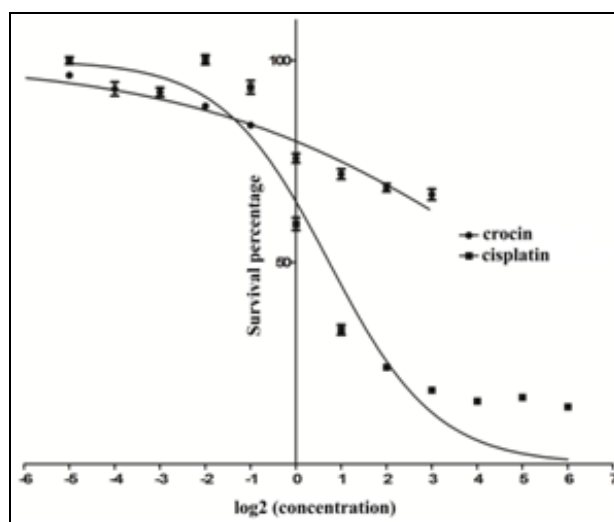


Fig. 1: The half maximal inhibitory concentration (IC₅₀) curve of BGC-823 cells after adding different concentrations of cisplatin and crocin for 48h. X axis represents the log₂ value of drug concentration. Y axis represents the survival percentage of tumor cells.

To confirm the inhibition effects of crocin, cisplatin and crocin plus cisplatin on BGC-823 cells, the inhibition rates were evaluated by MTT assay. As shown in table 1, the optical density (OD) values of crocin groups showed no significant differences (*p*> 0.05), whereas the cisplatin group showed significantly lower levels compared with the controls (*p*< 0.05). Furthermore, the combination of cisplatin with crocin significantly reduced tumor cell growth compared with cisplatin treatment alone (fig. 2),

and the inhibition rate was dose-dependent.

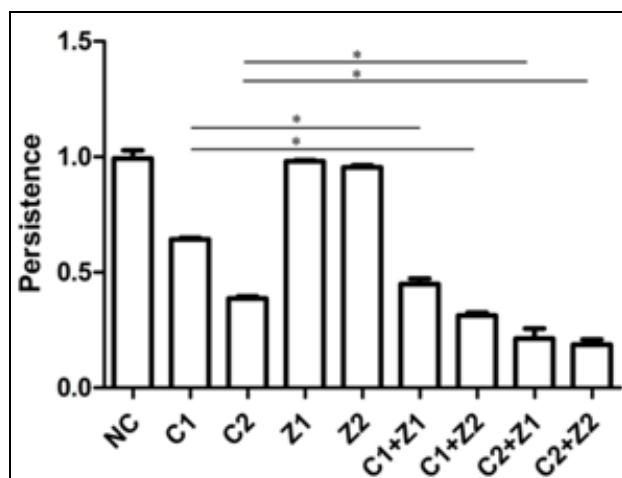


Fig. 2: The effect of cisplatin, crocin and cisplatin plus crocin on BGC-823 cell growth. C1 represents the 0.8 μ g/mL cisplatin, C2 represents the 1.6 μ g/mL cisplatin, Z1 represents the 8mg/mL crocin, and Z2 represents the 16mg/mL crocin.

Crocini potentiated cisplatin-induced tumor cell apoptosis

To determine whether cisplatin or crocin induces the BGC-823 cell apoptosis, the cells were treated with cisplatin, crocin and cisplatin plus crocin for 48h and the apoptosis analysis was performed by flow cytometry. The results showed that the apoptosis rates of cisplatin groups increased significantly compared with the control groups, while there was no significant difference between crocin groups and controls. However, crocin plus cisplatin significantly increased the apoptosis rates of BGC-823 cell in comparison with cisplatin treatment alone (fig. 3).

The combination of cisplatin with crocin increased p53 and Bax expression, decreased bcl-2 expression

Based on the results from qRT-PCR, the treatment of cisplatin significantly up-regulated the mRNA expression of *p53* and *Bax* ($p < 0.05$), but the levels of *Bcl-2* were similar to the controls (fig. 4). After being treated with crocin, the expression of *Bcl-2* was significantly up-regulated compared with controls ($p < 0.05$). In addition, the expression of *p53* and *Bax* in cisplatin plus crocin groups was significantly higher than those in controls and cisplatin group ($p < 0.05$). However, the expression of *Bcl-2* was significantly down-regulated in cisplatin plus crocin group ($p < 0.05$), as shown in fig. 4.

DISCUSSION

This is the first time demonstrating that the crocin enhances the effectiveness of cisplatin in gastric cancer in reducing proliferation of cancer cell and inducing apoptosis of tumor cells. In the present study, we found that crocin alone had no effect on the proliferation and

apoptosis of BGC-823 cells. This finding indicated that the cytotoxic effect of crocin on BGC-823 cells was low. However, the combination of cisplatin plus crocin had significantly cytotoxic effect on BGC-823 cells, with a dose dependent manner plus crocin. These results were in consistent with those from Hoshayar *et al.* (Hoshayar *et al.*, 2013) to some extents. Hoshayar *et al.* demonstrated that crocin alone presented both dose- and time-dependent cytotoxic effects against gastric adenocarcinoma cells and induced apoptosis. The difference might be caused by different sensitivity of various tumor cell lines to anticancer agents. Thus, further studies were still needed to investigate the causes of this difference.

It has been shown that cisplatin was used as a common chemotherapeutic agent for gastric cancer treatment (Pasini *et al.*, 2011). In this study, we confirmed that cisplatin inhibited gastric carcinoma cell proliferation and induced apoptosis of cancer cells by using MTT assay flow cytometry, which agreed with the results in previous studies (Sun Dong *et al.*, 2014). In addition, cisplatin often used as a adjunctive drug matching with other anticancer drugs to improve the therapeutic effect on gastric cancer (Van Cutsem *et al.*, 2006; KoizumiNarahara *et al.*, 2008). In our study, the combination of cisplatin with crocin enhanced the inhibition of tumor cell proliferation compared to cisplatin treatment alone, by significantly improving apoptosis rates. These evidences indicated that the combination of crocin with cisplatin in the treatment of gastric cancer was more effective than cisplatin alone, and thus the combination might be used as an alternative to cisplatin for chemotherapy for patients with gastric cancer.

To confirm the results from flow cytometry, we detected the expression patterns of apoptosis-related genes including *p53*, *BCL-2* and *Bax* using qRT-PCR technique. The results showed that the expression of *BCL-2* was in a reversed manner to *p53* and *Bax*, and crocin had a complementary function to cisplatin on the expression of *p53* and *Bax*, whereas cisplatin had a decisive effect on the *BCL-2* expression. Taken together, the results suggested that the ratios of *Bax/Bcl-2* in cisplatin plus crocin groups were significantly higher, whereas those in crocin groups were significantly lower than those in cisplatin and control groups ($p < 0.05$). These results were in accordance with results of MTT assay and flow cytometry analysis, demonstrating the complementary function of crocin to cisplatin.

The members of Bcl-2 family including anti-apoptotic proteins and pro-apoptotic proteins play crucial roles in apoptosis and are strongly associated with cancer therapy (Thomas *et al.*, 2013). Among these Bcl-2 members, Bcl-2 is an anti-apoptotic member which is elucidated in tumor development via dysfunction in apoptotic pathways

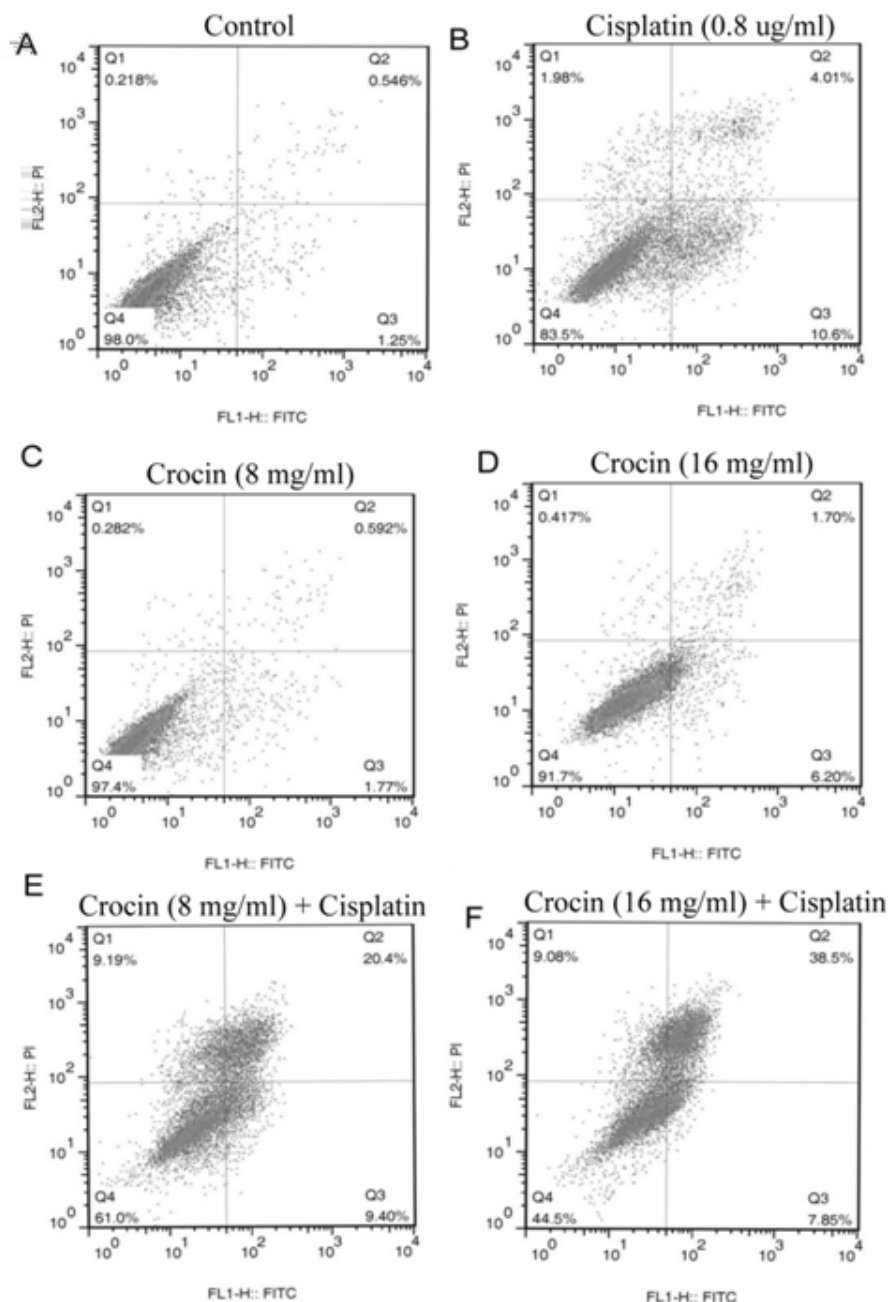


Fig. 3: Apoptosis rate analysis by flow cytometry. The BGC-823 cells were treated with crocin, cisplatin and cisplatin plus crocin for 48 h. Then the cells were harvested and were stained with FITC/PI and flow cytometry analysis was performed to analyze apoptosis rates.

(Adams and Cory, 2007). The pro-apoptotic gene *Bax* is related to a variety of apoptosis-related diseases such as cancer, and ratio of *Bcl-2* and *Bax* is considered important in determining the cell susceptibility or resistance to apoptosis (Ola *et al.*, 2011). Furthermore, alteration of the *Bax/Bcl-2* ratio has been reported to be associated with poor prognosis in tumors (Wei, 2004). Hoshyar *et al.* reported that crocin triggered the gastric adenocarcinoma cell apoptosis by increasing the *Bax/Bcl-2* ratio, in comparison with the human normal fibroblast skin cells

(HoshyarBathaie *et al.*, 2013). However, the results in this study suggested that the ratios of *Bax/Bcl-2* in crocin groups were significantly lower, whereas those in cisplatin groups and cisplatin plus crocin groups were significantly higher than those in controls ($p < 0.05$). This difference might be due to the cell types and the selection of negative controls, thus more experiments should be performed to confirm the effect of crocin on gastric carcinoma cell.

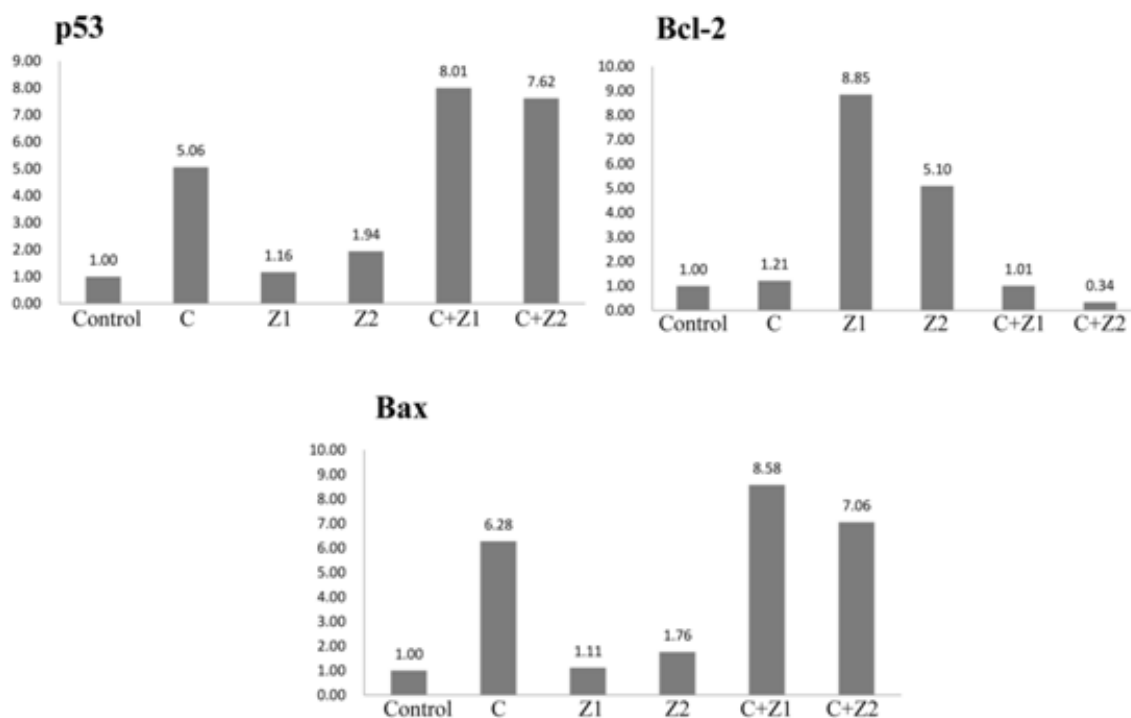


Fig. 4: The mRNA expression of *p53*, *Bax* and *Bcl-2* in BGC-823 cells after culturing with crocin, cisplatin and cisplatin plus crocin for 48h. C represents the 0.8 μ g/ml cisplatin, Z1 represents the 8mg/mL crocin, Z2 represents the 16mg/mL crocin.

Table 1: The proliferation inhibition of BGC-823 cells after treated with cisplatin, crocin and cisplatin plus crocin for 48h

Groups	Concentration	OD at 48h	Inhibition ratio
Black	-	0.007 \pm 0.003	-
Negative	-	0.694 \pm 0.043	-
Cisplatin	0.8 μ g/mL (C1)	0.454 \pm 0.038**	0.349
Cisplatin	1.6 μ g/mL (C2)	0.278 \pm 0.023**	0.594
Crocin	8mg/mL (Z1)	0.672 \pm 0.031	0.037
Crocin	16mg/mL (Z2)	0.642 \pm 0.037	0.078
Crocin + cisplatin	cisplatin 0.8 μ g/mL + crocin 8 mg/mL (C1+ Z1)	0.341 \pm 0.015**	0.509
Crocin + cisplatin	cisplatin 0.8 μ g/mL + crocin 16 mg/mL (C1+ Z2)	0.225 \pm 0.011**	0.676
Crocin + cisplatin	cisplatin 1.6 μ g/mL + crocin 8 mg/mL (C2+ Z1)	0.126 \pm 0.009**	0.821
Crocin + cisplatin	cisplatin 1.6 μ g/mL + crocin 16 mg/mL (C2+ Z2)	0.115 \pm 0.004**	0.832

** $p < 0.01$ VS negative control.

Furthermore, the *p53* is one of the best known tumor suppressor genes located at the short arm of chromosome 17, and acts as a key transcription factor. The *p53* is closely associated with the cell cycle regulation, DNA recombination, cell apoptosis and tumor suppression (Hofseth *et al.*, 2004; Li *et al.*, 2012). The *p53* regulates the apoptosis by interacting with the Bcl-2 family members. Recently, Hassan *et al.* (Hassan *et al.*, 2013) found that the *p53* is a negative regulator of *Bcl-2* and acts as a transcriptional activator of the *Bax*. In our study, the qRT-PCR results showed that cisplatin could promote the expression of *p53*, as expected. Moreover, the expression of *p53* and *Bcl-2* were changed in a reversed pattern, confirming that *p53* is a negative regulator of *Bcl-*

2, while as an activator of the *Bax*. When cisplatin was combined with crocin, the expression of *p53* was significantly up-regulated in comparison with other treatments ($p < 0.05$). These results showed that cisplatin alone or together with crocin might act as the activator of *p53* and *Bax* and then regulate the pathways they participate in.

In conclusion, our findings suggested that crocin in combination with cisplatin could suppress cell proliferation and induce apoptosis of BGC-823 cells. We speculated that crocin may be an effective complementary drug to cisplatin and the combination of crocin with cisplatin might be an alternative to cisplatin alone for

chemotherapy for patients with gastric cancer. However, more experiments in other models and clinical researches are still needed to confirm the function of crocin plus cisplatin *in vivo*.

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