

Crocetin inhibits pancreatic cancer cell proliferation and tumor progression in a xenograft mouse model

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Abstract

Crocetin, a carotenoid compound derived from saffron, has long been used as a traditional ancient medicine against different human diseases including cancer. The aim of the series of experiments was to systematically determine whether crocetin significantly affects pancreatic cancer growth both *in vitro* and/or *in vivo*. For the *in vitro* studies, first, MIA-PaCa-2 cells were treated with crocetin and in these sets of experiments, a proliferation assay using H³-thymidine incorporation and flow cytometric analysis suggested that crocetin inhibited proliferation. Next, cell cycle proteins were investigated. Cdc-2, Cdc-25C, Cyclin-B1, and epidermal growth factor receptor were altered significantly by crocetin. To further confirm the findings of inhibition of proliferation, H³-thymidine incorporation in BxPC-3, Capan-1, and ASPC-1 pancreatic cancer cells was also significantly inhibited by crocetin treatment. For the *in vivo* studies, MIA-PaCa-2 as highly aggressive cells than other pancreatic cancer cells used in this study were injected into the right hind leg of the athymic nude mice and crocetin was given orally after the development of a palpable tumor. The *in vivo* results showed significant regression in tumor growth with inhibition of proliferation as determined by proliferating cell nuclear antigen and epidermal growth factor receptor expression in the crocetin-treated animals compared with the controls. Both the *in vitro* pancreatic cancer cells

and *in vivo* athymic nude mice tumor, apoptosis was significantly stimulated as indicated by Bax/Bcl-2 ratio. This study indicates that crocetin has a significant antitumorigenic effect in both *in vitro* and *in vivo* on pancreatic cancer. [Mol Cancer Ther 2009;8(2):315–23]

Introduction

Adenocarcinoma of the pancreas, with an annual incidence of 9 to 10 cases per 100,000 per year, accounts for ~2% of all malignancies excluding basal and squamous cell cancers (1–3). It has a very poor prognosis, and the 2-year survival rate is <10% (3). Because of this high mortality rate, although it is relatively uncommon, pancreatic adenocarcinoma is the fourth leading cause of cancer death in the United States and results in an unaccounted number of additional deaths worldwide (4). Therefore, new therapeutic alternatives are desperately needed for patients with pancreatic adenocarcinoma, as nonsurgical therapies (chemotherapy, immunotherapy, radiation therapy, etc.) are largely ineffective, and metastatic disease frequently develops even after potentially curative surgery (1). Studies of the biology of pancreatic adenocarcinoma have revealed that the activation of specific oncogenes, growth factors, and transcription factors that are involved in regulation of inflammatory gene expression, play critical roles in its development and progression (1–4). Epidermal growth factor receptor (EGFR) also has an important role in the development, and increased expression of EGFR is detected in human pancreatic cancer (5, 6). Coexpression of EGFR and its ligands after activation of receptor-mediated signaling molecules is commonly linked with pancreatic cancer cell growth (7). Clinical studies have shown that coexpression of EGFR and EGF or transforming growth factor α is correlated with both an increase in tumor size and decreased patient survival (8). Recently, the combination of an EGFR antagonist when given with chemotherapy was found to be more effective than chemotherapy alone (9). Therefore, it is likely that future research will continue to focus on these basic cellular processes as the search continues for innovative therapeutics and prognostic markers to enhance survival of individuals with this malignancy.

The need for anticancer drugs with high efficacy and low toxicity has led to studies evaluating putative antineoplastic factors in fruits, vegetables, herbs, and spices. There are ancient reports of saffron being used to treat various diseases, particularly cancer, by the Indian, Greek, and Chinese cultures (10–24). Saffron, is present in the dry stigmas of the plant *Crocus sativus* L., and is used as a spice and a food colorant (10). Comprehensive chemical analysis of saffron extracts has shown that major constituents include carotenoids, and in particular, crocetin (14). Crocetin is an amphiphilic low-molecular weight

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carotenoid compound as shown in Fig. 1 (14). At the structural level, this molecule consists of a C-20 carbon chain with multiple double bonds, and a carboxylic acid group at each end of the molecule where R1 and R2 = H (14). A number of studies have reported an antitumor effect associated with saffron treatment in multiple cell culture systems and animal models (10–16). It was reported first in 1990s and confirmed in recent years that saffron extract inhibited growth of malignant cells *in vitro* and also *in vivo* (10–13). Inhibition of DNA, RNA, and protein synthesis was shown in three human malignant cells exposed to crocetin (11) and the mechanism of action was reported through suppression of the activity of DNA-dependent RNA polymerase II (16, 17). Another study also showed that crocetin reduces *in vitro* histone H1-DNA interaction and interfered with transcription (23). Crocetin and carotenoids in general, showed cytotoxic effects on a range of tumors and malignant cells (17, 18). During the last decade, a number of studies in animal model systems have shown an antitumor effect of saffron (19, 20). One *in vivo* study reported that crocetin has antitumor activity in a lung cancer animal model by scavenging free radicals and drug-metabolizing enzymes (15). In a recent *in vitro* study, crocetin showed significant reduction of cell proliferation in both MCF-7 and MDA-MB-231 breast cancer cells (24). Collectively, these studies provide strong evidence of the antitumor activity of crocetin. Given the potential importance of crocetin, this series of experiments was designed to examine the effect of crocetin on pancreatic adenocarcinoma cells and also to evaluate whether it has an antitumorigenic effect on pancreatic cancer in an athymic (nude) mice model.

Materials and Methods

Reagents

Anti-proliferating cell nuclear antigen (PCNA) and monoclonal antibodies of phospho-EGFR from Cell Signaling, EGFR from BD Biosciences, Bcl-2 from Calbiochem, Bax polyclonal antibodies from Santa Cruz Biotechnology, and β -actin monoclonal from Sigma Co. were obtained, respectively. A terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (DNA fragmentation detection kit) for histology was purchased from Oncogene Research. Peptamen was purchased from Nestle. Crocetin is purchased from MP Biomedical, and its purity is over 90% by high performance liquid chromatography.

Cell Lines, Culture Conditions, and Treatment

The human pancreatic adenocarcinoma cell lines MIA-PaCa-2, BxPC3, Capan-1, and ASPC-1 were obtained from the American Type Culture Collection and grown in DMEM (Sigma Co) supplemented with 1 mmol/L sodium pyruvate (Fisher Chemical Company), 100 U/mL of penicillin and 100 U/mL of streptomycin (Sigma Co), and 10% fetal bovine serum (HycloneT) at 37°C in a humidified tissue culture incubator containing 5% CO₂ and 95% air. Approximately 70% confluent cells were treated with different concentrations (50–200 μ mol/L) of crocetin for 72 h.

[³H] Thymidine Incorporation Assay. Pancreatic cancer cells were plated onto 24-well coming tissue culture plates. Cells were treated for 72 h with different concentrations (50–200 μ mol/L) of crocetin. [³H] Thymidine (Amersham Biosciences) was added to each control and treated samples placed in the wells at a rate of 1 μ Ci/mL (1 μ L/well) 18 h before harvesting the cells. The cells were washed by PBS and lysed with 0.25N NaOH. Lysates were transferred into vials containing a liquid scintillation cocktail (Perkin-Elmer) and radioactivity was measured by Packard Tri-Carb LS counter (GMI, Inc.). Triplicates were averaged and expressed as counts/min.

Flow Cytometric Analysis. After treatment with crocetin (50, 100, and 200 μ mol/L) for 72 h, MIA-PaCa-2 cells were collected by trypsinization, fixed in 70% ethanol, washed in PBS, and the cells were counted. After centrifugation, the cells were resuspended in PBS containing RNase (100 μ g/mL) 37°C for 30 min. After digestion of cellular RNA, cells were pelleted and resuspended in fresh PBS containing propidium iodide (0.5 mg/mL). Data were acquired on a FACS Scan flow cytometer (Becton Dickinson) and analyzed by using the Cellquest software.

Animals. Six- to 8-wk-old athymic female outbreed nude mice (nu/nu) were obtained from Charles River Laboratories and were used for tumor development. All animals were maintained in a sterile environment and daily 12-h light/12-h dark cycle. All the mice were maintained according to standard guidelines of American Association for the Accreditation of Laboratory Animal Care with the approval of the Institutional Animal Care and Use Committee of the Kansas City VAMC.

Tumor Production in Athymic Female Nude Mice. MIA-PaCa-2 cells (2.5×10^6) were injected into the right hind leg of each mouse for the development of tumor. The

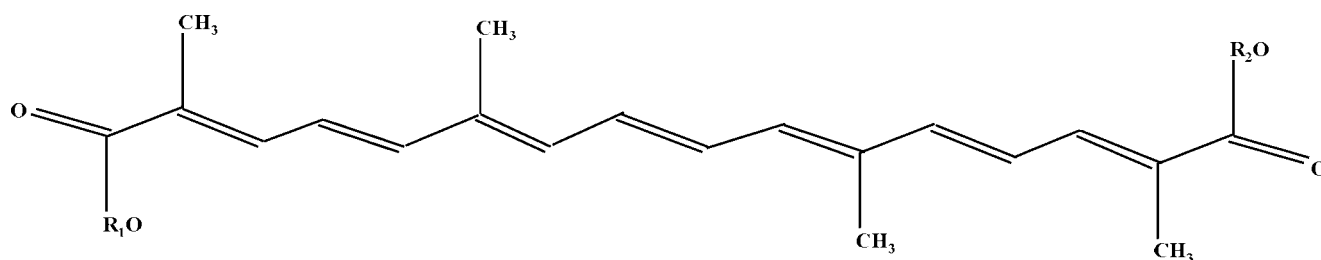


Figure 1. Known chemical structures of crocetin separated from Saffron by Tarantilis et al. (14).

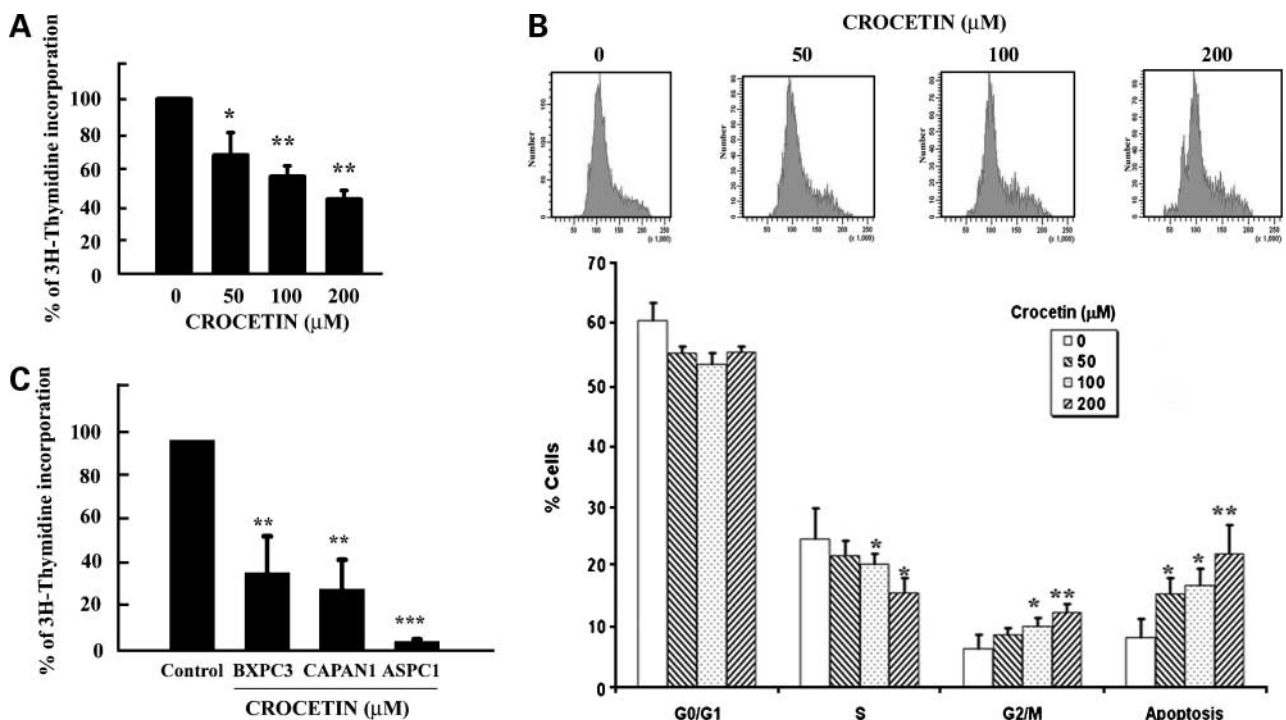


Figure 2. Effect on proliferation. **A**, MIA PaCa 2 cells were labeled with ³H-thymidine and incubated for 24 h. Crocetin at different concentrations (0, 50, 100, and 200 μmol/L) significantly could inhibit proliferation after 72 h of treatment compared with control. **B**, crocetin-induced cell cycle arrest and apoptosis in MIA-PaCa-2 cells using flow cytometry analysis. Crocetin-treated (50, 100, and 200 μmol/L) and vehicle-treated (0) cells were harvested and incubated with propidium iodide and analyzed for flow cytometry. *Bar diagram*, comparative cells population in G₀-G₁, S, G₂-M, and apoptotic cells in crocetin-treated cells. **C**, different pancreatic cancer cells: BxPC-3, Capan-1, and ASPC-1 cells were treated with 200 μmol/L of crocetin for 72 h and labeled ³H-thymidine as mentioned in **A**. Percentage of ³H-thymidine incorporation was significantly inhibited by crocetin treatment compared with untreated control shown as 100% in those cells. *Columns*, mean of three different experiments; *bars*, SD. *, *P* < 0.01; **, *P* < 0.001; ***, *P* < 0.0001 versus untreated control (Student's *t* test).

mice were divided into two groups (six mice per group) with a control group and crocetin treatment group. After development of palpable tumor, the tumor size was measured twice in a week. When tumors are well-established (~0.34 cm³), treatment was started. The treatment group received crocetin at 4 mg/kg/d orally dissolved in 15 mL of peptamen for 12 h during dark for 30 d, and the dose is in agreement with other investigators (14, 17, 20, and 27). Control group received only peptamen. The mice were given solid diet (Harlem TEKLAD Rodent Diet) during light time after liquid peptamen diet at dark. Tumor size was monitored twice weekly, and its volume calculated as $V = (a \times b)^2/2$, where *a* is the length and *b* is width. All mice were sacrificed after 30 d after treatment, and tumors were taken out and stored at -80°C.

Western Blot Analysis. The immuno-Western blot analyses were same as described earlier by Banerjee et al. (25). Briefly, MIA-PaCa-2 and BxPC-3 cells after treatment with or without crocetin and tumor samples were homogenized. Then both cell and tumor lysates were spun at 18,000 *g* for 1 h at 4°C, and the supernatants were collected for immunodetection. Protein concentration was measured by using the Comassie blue reagent assay (Pierce, Rockford, IL). Equal amount of protein were

resolved in 10% SDS-PAGE, transferred onto nitrocellulose membranes. Membranes were blocked with super block (Pierce) followed by incubation with appropriate primary antibodies at 4°C, overnight. The antigen-antibody reactions were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG for 1 h in room temperature. Immunoreactions were visualized by the enhanced chemiluminescence reagent kit (Pierce), and relative expression of proteins were calculated by densitometric analyses using ID image Analysis Software version 3.6 (Eastman Kodak Company).

Immunohistochemistry. Immunohistochemical analysis was done according to our previous described method (26) using Zymed broad range immunohistochemical kits (Zymed Laboratories). Tumor tissue samples were fixed in 4% buffered formalin and embedded in paraffin. The serial sections of 5 μm were cut. The tissue sections were deparaffinized and were hydrated in different concentrations of ethanol (i.e., 100%, 90%, 70%, and 50%). Endogenous peroxidase activity was blocked at room temperature by incubating in hydrogen peroxide in methanol (1:9). Slides were washed with PBS and nonspecific binding was blocked by blocking solution for 10 min. The sections were incubated with respective primary antibody in a

humidified chamber at 4°C overnight. The tissues were incubated with biotinylated secondary antibody (Zymed kit). The slides were then incubated with horseradish peroxidase–linked enzyme conjugate for 10 min. Positive reactions were visualized by incubating slides with stable 3,3'-diaminobenzidine solution (Zymed kit) and counterstained with hematoxylin. The slides were dehydrated and mounted in permount for microscopic examination.

Histochemical TUNEL Assay. To detect apoptosis, the terminal deoxynucleotidyl transferase–FragEL DNA Fragmentation Detection kit from Oncogene was used. The tumor tissue sections (5 μm) were rehydrated and permeabilized with proteinase K. Endogenous peroxidase was inactivated with 3% H₂O₂ at room temperature for 5 min. The slides were incubated with equilibrium buffer at room temperature for 30 min followed by 1.5 h of incubation with the terminal deoxynucleotidyl transferase labeling reaction mixture at 37°C. A stop/wash solution for 5 min at 37°C terminated the reaction, after which the tissues rinsed with TBS. The slides then were incubated with streptavidin peroxidase conjugate for 30 min at room temperature after which the slides were stained with 3,3'-diaminobenzidine solution and methyl green stain for color development.

Statistical Analysis. All the experiments were done in triplicate for each of the observations. Each of the data

represents the mean ± SD from three separate experiments. Statistical analysis was done between the groups of data by paired Student's *t* test. *P* values of <0.05 were considered as statistically significant.

Results

Effect of Crocetin on Proliferation and Cell Cycle Arrest *In vitro*

MIA-PaCa-2 cells first were treated with crocetin (50–200 μmol/L) for 72 hours. Treated cells were then labeled with ³H-thymidine and incubated for 18 to 20 hours. Cell proliferation was inhibited with all concentrations of crocetin as evaluated by the thymidine incorporation assay and the inhibition was 71%, 59%, and 43% in 50, 100, and 200 μmol/L concentrations, respectively, showing more pronounced inhibition of proliferation at the higher concentration (Fig. 2A).

To understand the mechanism responsible for crocetin mediated cell growth inhibition, the cell cycle distribution was evaluated using flow cytometric analysis. Results in Fig. 2B showed that there is also a significant accumulation of MIA-PaCa-2 cells in the G₂-M phase and this effect is highly significant in both the 100 and 200 μmol/L concentrations. There is a significant reduction of cells in S phase, whereas accumulation of cells in sub-G₀ or

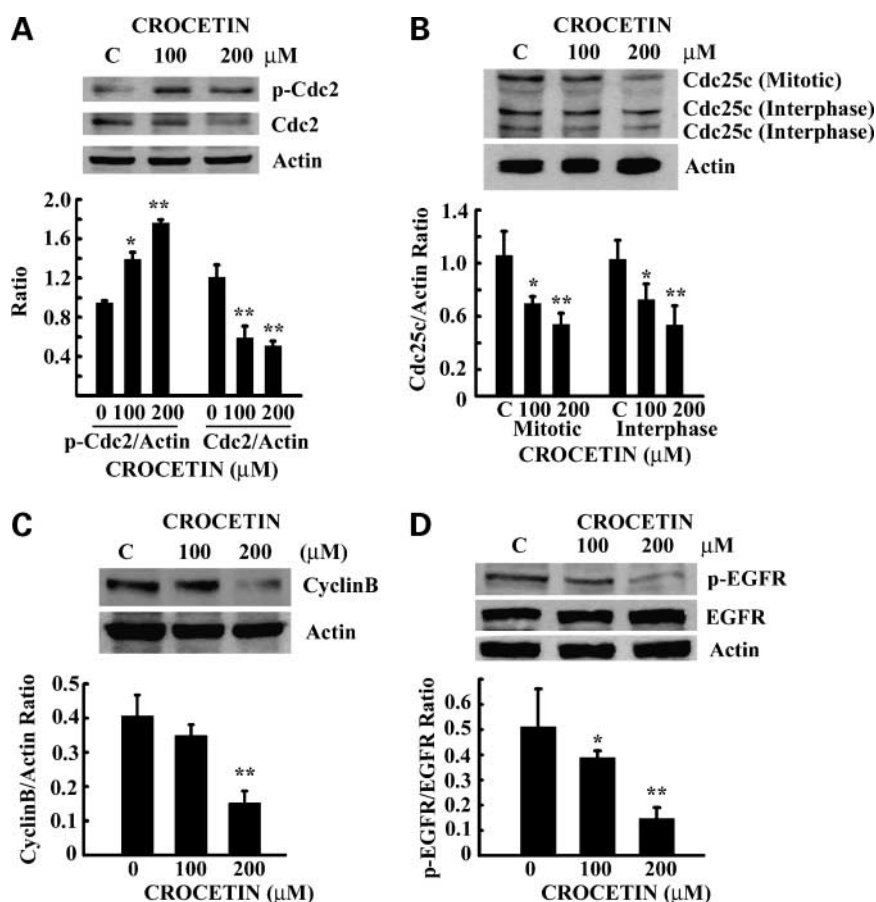


Figure 3. Crocetin effect on growth-related protein expression. **A**, expression of Cdc-2: Pancreatic cancer cells are treated with crocetin (100 and 200 μmol/L) for 72 h. Then the cells were lysed and centrifuged for subcellular fractions after treatment. The proteins from the fractions were separated by SDS-PAGE and transferred to nitrocellulose membranes. Phosphorylated (p) Cdc-2 protein was analyzed by Western Blot using pCdc-2 antibody as described in Materials and Methods section using densitometric analysis. The results of crocetin treated were compared with untreated control (C). **B**, expression of cdc-25c: Cells were treated similarly as mentioned in Fig. 3A and Western Blot was done using pCdc-25c antibody as described in Materials and Methods section. **C**, expression of Cyclin B1: Cells were treated similarly as mentioned in Fig. 3A and Western Blot was carried out using Cyclin B1 antibody as described in Materials and Methods section. **D**, EGFR expression: MIA PaCa-2 cells are treated with 100 and 200 μmol/L crocetin with appropriate control as described in **A**. The cells were prepared for Western blot analysis of phosphorylated and total protein of EGFR and expressed as ratio between them normalized with β-actin. Columns, mean of three different experiments; bars, SD. *, *P* < 0.01; **, *P* < 0.001; ***, *P* < 0.0001 versus untreated control (Student's *t* test).

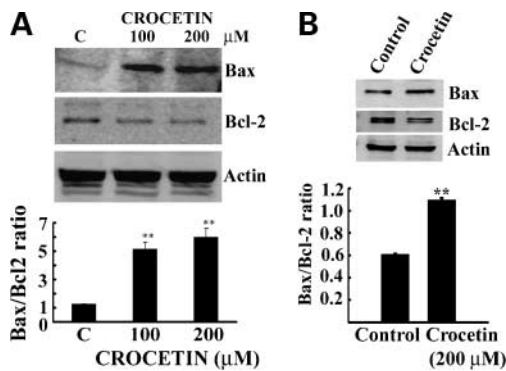


Figure 4. Crocetin effect of apoptotic proteins. **A**, Bax/Bcl-2 expression in MIA-PaCa-2: cells were treated with crocetin as described in Materials and Methods section and apoptotic proteins, Bax, and Bcl-2 were analyzed by Western blot as mentioned in Fig. 3A. **B**, Bax/Bcl-2 expression in BxPC-3: BxPC-3 cancer cells were treated with crocetin (200 μmol/L) for 72 h, and Bax and Bcl-2 were analyzed similarly as mentioned in Fig. 3A. Columns, mean from three different experiments; bars, SD. *, $P < 0.05$; **, $P < 0.001$, versus untreated control (Student's t test).

apoptosis phase was observed after crocetin treatment. The accumulation of apoptotic cells was highly significant with increasing concentrations of crocetin. Therefore, those two concentrations (100 and 200 μmol/L) are used for next phase of studies and those concentrations are in agreement with the study of other investigators (11, 12, 24).

To confirm further the effect of crocetin on pancreatic cancer cells, BxPC-3, Capan-1, and ASPC-1 were next treated with crocetin. In these experiments, crocetin 200 μmol/L was used because MIA-PaCa-2 showed significant inhibition in that dose. Crocetin (200 μmol/L) significantly inhibited the proliferation in all those pancreatic cancer cell lines. ASPC-1 was affected most by crocetin treatment (Fig. 2C).

Effect of Crocetin on Growth Regulatory Proteins and Apoptosis *In vitro*

To understand molecular changes involved in proliferation, cell cycle regulatory proteins are evaluated next. Because crocetin inhibited cell cycle progression at the G₂-M phase, we first investigated one of the key cell cycle proteins, Cdc-2. Cdc-2 phosphorylation inhibits cell differentiation at the G₂-M phase, which results cell arrest at G₂-M and that event is regulated by Cdc-25c phosphatase (27). The data presented in Fig. 3A shows Cdc-2 phosphorylation significantly increased 2-fold with crocetin treatment in both 100 and 200 μmol/L concentrations compared with control followed by a significant decrease in Cdc-2 expression.

To understand further, Cdc-25c phosphatase was examined and Fig. 3B revealed that Cdc-25 phosphatase expression in both mitotic and interphase proteins was inhibited significantly, suggesting the increased Cdc-2 phosphorylation correlated with a decreased expression of Cdc-25c phosphatase. Next, Cyclin B1 was evaluated because Cyclin B1 has been considered as marker of interphase including S and G₂ phases (27–29). The data shown in Fig. 3C shows that Cyclin B1 is significantly inhibited after crocetin treatment.

Next, EGFR protein phosphorylation and its expression were investigated because EGFR status has been correlated with the growth of the tumor cells (5–8). Western blot analysis in Fig. 3D showed that crocetin significantly inhibited EGFR activity, in both concentrations (100 and 200 μmol/L), as indicated by the ratio between the phosphorylated and unphosphorylated protein. This change was ~2- to 3-folds in the MIA-PaCa-2 cells compared with the untreated control.

To explore further the antitumorigenic effect of crocetin, apoptosis was investigated using Bax/Bcl2 expression.

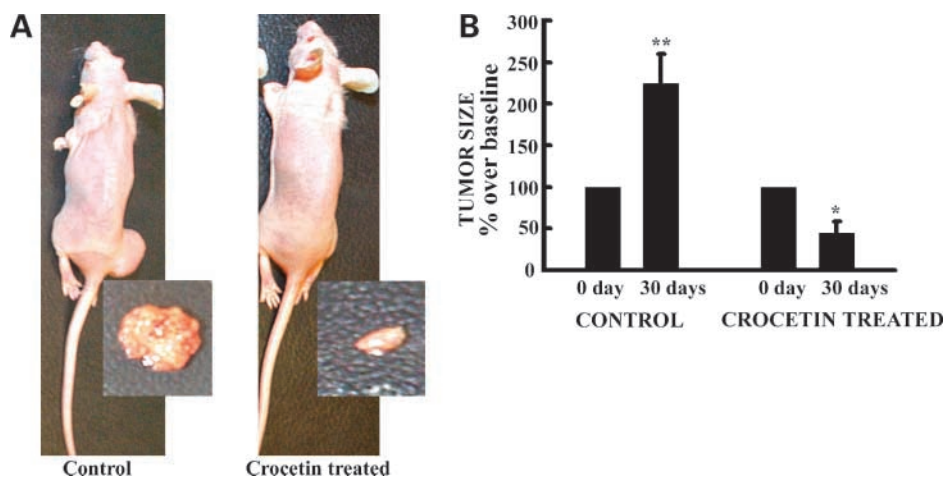


Figure 5. Crocetin inhibits pancreatic tumor formation in nude mice. MIA-PaCa-2 cells (2.5×10^6) were injected into athymic (nude) mice. After a 30-d period during which palpable tumors developed, animals were then treated with crocetin (4 mg/kg) given in the diet (peptamen) for 30 d. The animals were euthanized and tumors were excised and measured. The tumor size [Volume = $(a \times b)^2/2$, where a is length and b is width] was measured from the start date of crocetin treatment (0 d) to end date of crocetin treatment (30 d) compared with control (no treatment). Number of animals is six in both control and treated group. The tumor size as shown in the insets of the xenograft tumors removed from the respective mice (**A**) and percentage of tumor growth was presented in the graph after crocetin treatment (**B**). Columns, mean of six animals in each group; bars, SD. **, $P < 0.01$; ***, $P < 0.001$ versus untreated control (Student's t test).

Crocetin-treated MIA-PaCa-2 cells showed an increased Bax expression with concomitant decrease of Bcl-2 protein with the resultant enhancement of the ratio (4–5 folds in both concentrations) of Bax/Bcl-2 (Fig. 4A). To confirm further the effect of crocetin on apoptosis, BxPC-3 pancreatic cancer cells treated with crocetin (200 $\mu\text{mol/L}$) showed significant increase of Bax/Bcl-2 ratio (Fig. 4B).

Effect of Crocetin on Tumor Regression in Athymic (nude) Mice

Next, pancreatic cancer cells particularly MIA-PaCa-2 were used for *in vitro* studies because MIA-PaCa-2 cells are characteristically more aggressive than other pancreatic

cancer cells (BxPC-3, Capan-1, and ASPC-1) and have the ability to develop tumors within several weeks of inoculation (26). MIA-PaCa-2 cells were introduced in the athymic nude-mice for 3 to 4 weeks until palpable tumor was formed. Then, the mice (6) in each group were treated with or without crocetin (4 mg/kg/day) for 30 days and the tumors were measured twice per week until the mice were sacrificed. The tumor incidence was 100% in all the animals. The growth of the tumors was significantly regressed in crocetin-treated animals ($45\% \pm 10.5\%$) as comparison to vehicle treated control ($225\% \pm 36\%$) animals ($P < 0.001$; Fig. 5).

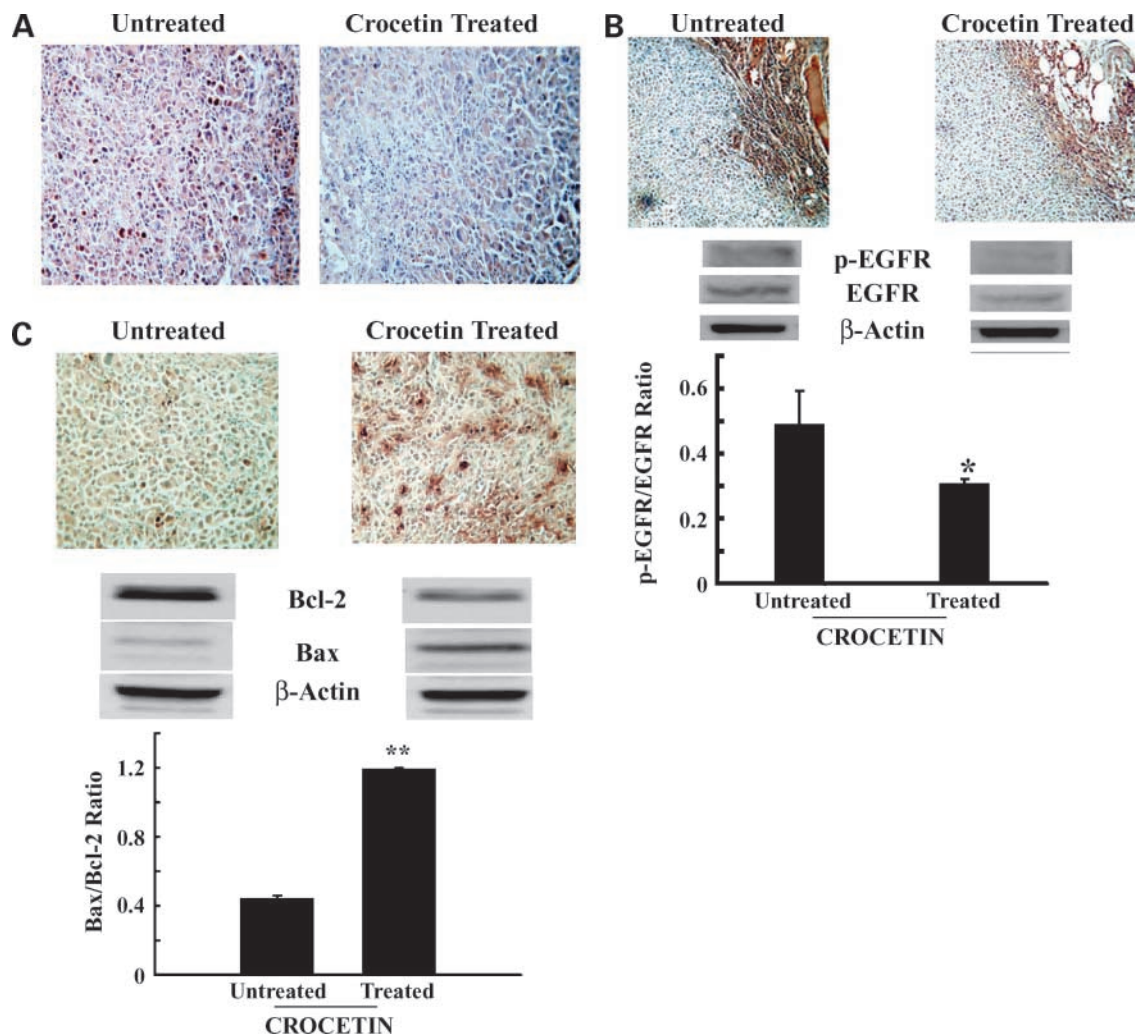


Figure 6. Crocetin effect on proliferation and apoptosis in xenograft tumors. **A**, expression of PCNA: Cell proliferation in tumor tissues, obtained from athymic mice as depicted in Fig. 5, was evaluated by staining for PCNA using histochemical analysis. PCNA expression, as indicated by the dense red collections of stain in untreated, was significantly decreased in crocetin-treated tumors, indicating decreased proliferation associated with crocetin treatment. **B**, expression of EGFR: Tissue sections were obtained from human pancreatic cancer tumors growing in nude mice as described in Fig. 5 and stained for EGFR as described in Materials and Methods section (top). Middle, EGFR expression of pancreatic tumors from same crocetin-treated and untreated control animals using Western Blot as described in Fig. 3A was determined. Phosphorylated (p-EGFR) and total protein of EGFR expressed as ratio between them normalized with β -actin was shown (bottom). **C**, Apoptosis: Apoptosis was evaluated by TUNEL assay using FragEL kit. Top, tumor tissues were processed for TUNEL assay. Increased apoptosis in pancreatic cancer xenografts tumors from animals receiving daily crocetin compared with pancreatic cancer xenografts receiving vehicle as control. Bax and Bcl-2 (middle) expressions of pancreatic tumors from same crocetin-treated and control animals using Western Blot were carried out and Bax/Bcl-2 ratio was also determined (bottom). Columns, mean from six different animals; bars, SD (**B** and **C**). *, $P < 0.05$; **, $P < 0.001$ versus untreated control (Student's *t* test).

Effect of Crocetin *In vivo* on PCNA, EGFR Expression, and Apoptosis

To explore whether the regression of tumor growth by crocetin is due to inhibition of proliferation, apoptotic cell death, or both, we first evaluated PCNA expression and then EGFR expression in tumors using histochemical analysis. As shown in Fig. 6A, PCNA-positive cells were elevated significantly in untreated samples compared with crocetin-treated samples. EGFR expression and phosphorylation was significantly reduced in crocetin-treated animals when compared with the untreated samples. Additionally, EGFR activity as determined by the ratio of EGFR phosphorylated form and unphosphorylated form was significantly impaired in the tumors due to crocetin treatment (Fig. 6B). Next, apoptosis by TUNEL assay was investigated in the tumors of mice treated with or without crocetin. TUNEL assay-positive cells were elevated markedly in crocetin-treated samples compared with untreated samples (Fig. 6C).

Collectively, these sets of experiments indicate that inhibition of tumor growth is due to the induction of apoptosis as well as inhibition of proliferation. To further confirm the apoptotic effect, the Bax/Bcl-2 ratio was evaluated. As shown in Fig. 6C, expression of Bax protein was increased with a concomitant decrease of Bcl-2 protein.

Discussion

Pancreatic cancer is the fourth leading cause of cancer death in the Western world (4, 30). Pancreatic tumors are highly resistant to current available therapies and the 5-year survival is dismal with a median expected post-diagnosis survival time of 5 months (30, 31). Owing to poor prognosis, alternative therapies are being investigated. Crocetin, a carotenoid compound derived from saffron, has shown a significant inhibitory effect on the growth of cancer cells (14, 15, 18). Potential mechanisms for crocetin-mediated inhibition of tumor growth include the reduction in the synthesis of DNA, RNA, and protein (17, 18). It has also been shown that crocetin inhibits RNA polymerase II activity (16, 17). Crocetin also interferes with histone H1 structure and H1-DNA interactions, suggesting for another possible mechanism of anticarcinogenic action (23). The exact mechanism of the protective activity of crocetin is not clear at present, but several hypotheses have been forwarded, which suggest that carotenoids can convert to vitamin A, can enhance carcinogen metabolism, can act as an antioxidant or can inhibit nucleic acid synthesis (11–13, 31). In the present study, the proliferation of pancreatic adenocarcinoma cells is significantly inhibited due to crocetin treatment. Similarly, pancreatic cancer growth in nude mice was also significantly inhibited due to the oral administration with crocetin. Therefore, it was imperative to investigate the effect of crocetin on the proliferation of pancreatic cancer cells and pancreatic tumor regression in nude mice.

In the present study, treatment of pancreatic cancer cells with crocetin significantly inhibited cell distribution in the

S phase ensuring impairment in DNA replication (32) confirming the inhibition of DNA synthesis in crocetin-treated pancreatic cancer cells and the increased accumulation of cells in the G₂-M phase. The entry of cells in the eukaryotic system depends upon the activity of several regulatory proteins including Cdc-2, Cdc-25C, Cyclin B1, and other proteins (27–31). Cdc2, one of the checkpoint proteins that regulate the G₂-M phase in the cell cycle, was evaluated first because the phosphorylation of Cdc-2 is required to activate some checkpoint proteins for maintenance of the sequential progression of cell cycle (27). The phosphorylation of Cdc-2, which in turn regulates the cell division cycle at the G₂-M phase, showed a significant increase with crocetin treatment. The hyperphosphorylation of Cdc-2 at tyrosine residues inactivates the kinase activity and that results in the inhibition of entry of cells from G₂ to mitosis (27). Although there is significant hyperphosphorylation of Cdc-2 at tyrosine residues, the levels and expression of Cdc-2 are significantly reduced due to crocetin treatment. These observations suggested that the subsequent inactivation of kinase activity due to crocetin treatment is largely associated with the expression and levels of Cdc-2 (27, 33). The increase of Cdc-2 phosphorylation and inhibition of kinase activity with resultant cell cycle arrest could be important events for the inhibition of proliferation in crocetin-treated pancreatic cancer cells. It seems that the crocetin-treated pancreatic cancer cells arrest of the cell cycle is directly due to the stimulation of the phosphorylation of Cdc-2, which in turn inactivates the Cdc-2 protein itself ultimately inhibiting proliferation and growth. With this in mind, Cdc-25c phosphatase was evaluated because it plays critical role in regulating Cdc-2 phosphorylation (31). The inhibition of Cdc-25c expression suggested an increase of Cdc-2 phosphorylation, which could be the basis for the arrest of cells in the G₂-M phase (31, 32, 34, 35). Three major forms of Cdc-25c have been identified (57, 60, and 80 kDa) that are involved in asynchronous dividing cells (36). The 57- and 60-kDa protein bands are interphase form and the 80-kDa band is a mitotic form. The inhibition of both interphase and mitotic forms, which suggests a prevention of dephosphorylation of Cdc-2, maintains arrest in the G₂-M phase (37–40). The eukaryotic cell cycle progression involves sequential activation of cyclin-dependent kinases whose activation is dependent on their association with cyclins (41, 42). A complex formed by the association of Cdc2 and Cyclin B1 plays a major role at the entry of mitosis (42). Therefore, the expression of Cyclin B1 was evaluated and showed an inhibition of expression of Cyclin B1. Therefore, the inhibition of Cyclin B1 along with the inactivation of Cdc2 could be the major event for leading to crocetin induced G₂-M phase arrest. Overall, our studies evaluating these major cell cycle checkpoint proteins indicate crocetin is effective as antiproliferative agent accomplishing cell cycle arrest.

EGFR is another critical regulator of cellular proliferation and differentiation and plays a central role in tumor proliferation and growth (5–8). Crocetin significantly

reduce EGFR expression and phosphorylation as indicated by the ratio in pancreatic cancer cells. Immunohistochemical and Western blot analysis revealed that a significant decrease of EGFR phosphorylation and expression in the tumors, which developed in nude mice after crocetin treatment. These studies indicated that crocetin is a very effective inhibitor of EGFR activity and that inhibition correlates with impaired growth.

Crocetin showed cytotoxicity to tumor cells (10, 11, 14), therefore, it is plausible that it stimulated apoptosis or programmed cell death. Bcl-2, an antiapoptotic protein, can be used to measure levels of tissue apoptotic cell death. Bcl-2 protein is known to inhibit apoptosis induced by a variety of physiologic and pathologic stimuli (43, 44). Bax has a proapoptotic effect and also counters antiapoptotic effect of Bcl-2 (43, 44). It has been proposed that the ratio of Bax/Bcl-2 may govern the sensitivity of cells of apoptotic stimuli (45, 46). In this study, the ratio of Bax/Bcl-2 was significantly increased in both pancreatic cancer cells as well as in pancreatic tumors after crocetin treatment, which suggested that the reduction on imbalance between antiapoptotic (Bcl-2) and proapoptotic (Bax) could be a major factor in antitumor activity of crocetin.

As suggested by our observations on the inhibition of proliferation and growth due to treatment of crocetin both *in vitro* and *in vivo*, the potential utility of crocetin is very much similar in both *in vitro* and *in vivo*. The dosages of crocetin used *in vitro* and *in vivo* studies are also comparable as suggested by other investigators (12, 18, 22). It is also noteworthy that crocetin was used *in vivo* longer periods of time in this study, whereas it was used *in vitro* until 72 h. Another major problem in the use of anticancer agents is their toxic effect to normal cells. The concentrations of crocetin used in both *in vitro* and *in vivo* study are relatively nontoxic to human cells (10, 11, 15, 22, 24). It was reported previously the LD₅₀ of crocetin is very high 2 g/kg (10, 12, 24). It has also been suggested that carotenoids are well-tolerated at high doses and numerous studies have supported their use in cancer chemoprevention and chemotherapy (12, 13). Therefore, raising the possibility that crocetin could be relatively nontoxic with a potential to exert an antitumor effect. In summary, our study showed that crocetin down-regulated growth and proliferation stimulated apoptosis and resulted in significant growth regression in *in vivo* pancreatic tumors. At present, it is not known that the effect of crocetin on pancreatic cancer regression is its own receptor-dependent or receptor-independent mechanisms, which will be investigated in future to understand exact mechanism of crocetin action.

In conclusion, this study indicated for the first time that crocetin could be used as a novel therapy for pancreatic cancer due to significant antitumor effect and thereby, can be of great therapeutic benefit in future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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