Crocetin induces cytotoxicity and enhances vincristine-induced cancer cell death via p53-dependent and -independent mechanisms

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Aim: To investigate the anticancer effect of crocetin, a major ingredient in saffron, and its underlying mechanisms.

Methods: Cervical cancer cell line HeLa, non-small cell lung cancer cell line A549 and ovarian cancer cell line SKOV3 were treated with crocetin alone or in combination with vincristine. Cell proliferation was examined using MTT assay. Cell cycle distribution and sub-G1 fraction were analyzed using flow cytometric analysis after propidium iodide staining. Apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit with flow cytometry. Cell death was measured based on the release of lactate dehydrogenase (LDH). The expression levels of p53 and p21WAF1/Cip1 as well as caspase activation were examined using Western blot analysis.

Results: Treatment of the 3 types of cancer cells with crocetin (60–240 μmol/L) for 48 h significantly inhibited their proliferation in a concentration-dependent manner. Crocetin (240 μmol/L) significantly induced cell cycle arrest through p53-dependent and -independent mechanisms accompanied with p21WAF1/Cip1 induction. Crocetin (120–240 μmol/L) caused cytotoxicity in the 3 types of cancer cells by enhancing apoptosis in a time-dependent manner. In the 3 types of cancer cells, crocetin (60 μmol/L) significantly enhanced the cytotoxicity induced by vincristine (1 μmol/L). Furthermore, this synergistic effect was also detected in the vincristine-resistant breast cancer cell line MCF-7/VCR.

Conclusion: Crocetin is a potential anticancer agent, which may be used as a chemotherapeutic drug or as a chemosensitizer for vincristine.

Keywords: crocetin; vincristine; cell cycle; apoptosis; p53; neoplasm
malignant cells including human rhabdomyosarcoma (RD) cells\textsuperscript{30}, pancreatic cancer cells\textsuperscript{11}, and breast cancer cells\textsuperscript{12} in in vitro studies. In the pancreatic cancer xenograft mouse model, significant regression in tumor growth with inhibition of proliferation and enhanced apoptosis was observed in crocetin-treated animals compared with the control animals\textsuperscript{11}. In addition, crocetin inhibits 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin tumors in the tobacco-specific carcinogen benzo(a)pyrene [B(a)P] initiated mice\textsuperscript{33} and exhibits protective effects against B(a)P-induced lung carcinogenesis\textsuperscript{14}. Collectively, these studies provide evidence of the antitumor activity of crocetin.

Saffron is expensive and has limited availability. To avoid high costs of saffron, Prof Yong QIN’s laboratory (Sichuan University, China) has successfully synthesized crocetin using total synthesis. Given the importance of crocetin as a potential anticancer agent, the present study was designed to examine the effect of crocetin on several types of human cancer cells. Crocetin inhibited cell proliferation by inducing cell cycle arrest in cancer cells that were derived from the cervix (HeLa), ovary (SKOV3), and lung (A549) through p53-dependent and -independent mechanisms. Crocetin induced cytotoxicity in these cancer cells by enhancing apoptosis in a time-dependent manner. In the present study, we demonstrated that crocetin significantly sensitized these cancer cells to vincristine-induced cell death and that the synergistic effect was detected in a vincristine-resistant breast cancer cell line. Altogether, this study suggests that crocetin is a potential chemopreventive agent and a potential anticancer agent that can be used as a chemotherapeutic drug or a chemosensitizer for vincristine.

The vincristine-resistant mammary cancer cell line MCF-7/VCR was obtained from the Immunology Department at the West China School of Preclinical and Forensic Medicine at Sichuan University. MCF-7/VCR cells are cross-resistant to doxorubicin and were grown in RPMI-1640 containing 10% FBS and 1 μmol/L vincristine under standard conditions. The cells were cultured in the absence of vincristine one week before performing experiments.

**Materials and methods**

**Reagents**

Crocetin was kindly provided by Prof Yong QIN’s laboratory (Sichuan University, Chengdu, China). Vincristine (VCR), propidium iodide (PI), and MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) were purchased from Sigma (St Louis, MO, USA). The pan-caspase inhibitor Z-VAD-FMK was purchased from Calbiochem (La Jolla, CA, USA). Antibodies against active caspase-3, poly(ADP-ribose) polymerase (PARP) were purchased from BD Biosciences (San Diego, CA, USA). Anti-caspase-9 and anti-p53 were purchased from Cell Signaling (Beverly, MA, USA). Anti-β-actin was purchased from Proteintech (Chicago, IL, USA). Anti-p21\textsuperscript{WAF1/Cip1} was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Cell culture**

The cervical cancer cell line HeLa, non-small cell lung cancer cell line A549, and ovarian cancer cell line SKOV3 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were grown in RPMI-1640 or DMEM supplemented with 10% fetal bovine serum (FBS), 1 mmol/L glutamate, 100 units/mL penicillin, and 100 μg/mL streptomycin and cultured in an incubator (37°C, 5% CO\textsubscript{2}).

The MTT assay

Cell proliferation was measured by MTT assays as previously described\textsuperscript{35}. Briefly, cells were treated with different doses of crocetin for 48 h. Treated cells were incubated with MTT for approximately 3 h, rinsed two times with phosphate buffered saline (PBS), and dissolved with DMSO. The absorbance of the samples was measured at 570 nm using a plate reader. All the experiments were repeated three times, and the averaged result is shown in each figure.

**Flow cytometric analysis**

Flow cytometry was used to analyze the cell cycle distribution. After being treated as indicated in each figure legend, the cells were trypsinized, washed once with PBS, and fixed in 70% ethanol on ice for 1 h. Fixed cells were resuspended in PBS containing RNase (100 μg/mL) at 37 °C for 30 min. After digestion of cellular RNA, the cells were stained with propidium iodide (PI) staining solution (0.2% FBS and 25 μg/mL PI in PBS) at room temperature for 1 h in the dark. The stained cells were analyzed using a FAScan flow cytometer (Beckman Coulter Cell). To assess apoptosis, HeLa cells were treated with crocetin as indicated in each figure legend and double stained with Annexin V-FITC and PI using the Annexin V-FITC Apoptosis Detection Kit (Nanjing KeyGen Biotech, Nanjing, China) followed by flow cytometric analysis. Early apoptosis was defined by Annexin V\textsuperscript{+}/PI\textsuperscript{−} staining (Q4), and late apoptosis was defined by Annexin V\textsuperscript{+}/PI\textsuperscript{+} staining (Q2).

**Cell death assay**

After each designated treatment, cell death was detected based on the release of lactate dehydrogenase (LDH) using a cytotoxicity detection kit (Promega, Madison, WI, USA) as previously described\textsuperscript{36}. All of the experiments were repeated three to five times, and the averaged result is shown in each figure.

**Western blot analysis**

Cell lysate was collected by lysing cells in M2 lysis buffer [20 mmol/L Tris-HCl (pH 7.6), 0.5% NP40, 250 mmol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, and 1 μg/mL leupeptin]. Cell extracts were subjected to SDS-PAGE and analyzed by Western blot. The proteins were visualized by enhanced chemiluminescence (Millipore, Billerica, MA, USA) using the BIO-RAD Image station. Each experiment was repeated at least three times, and representative results are shown.
Statistical analysis
The data were represented as the mean±standard deviation (SD) from at least three independent experiments. The 95% confidence limits of the IC50 values were calculated. Comparisons between groups were performed by paired Student’s t test using the SPSS statistics software package (IBM SPSS, Chicago, IL, USA). P<0.05 was used for statistical significance.

Results
Crocetin inhibits cell proliferation in cancer cells
Crocetin inhibits cell proliferation in pancreatic adenocarcinoma cells and breast cancer cells[11, 12]. We first tested the effect of crocetin on several cancer cell types to determine whether the antiproliferative effect was common in different cancer cell types. We first treated cervical cancer HeLa cells with different doses of crocetin for 48 h. The MTT assay was used to measure viable cells. As shown in Figure 1A, crocetin reduced viable cell numbers in a dose-dependent manner, showing a pronounced effect at the higher concentration, i.e., with a reduction of viability of approximately 80% at a concentration of 240 μmol/L crocetin. Under these experimental conditions, marginal cell death was observed. The reduction of viable cell numbers detected by the MTT assay was mainly attributed to inhibition of cell proliferation. Similar dose-dependent anti-proliferation effects of crocetin were observed in non-small cell lung cancer A549 cells and ovarian cancer SKOV3 cells (Figure 1B and 1C). The IC50 values and 95% confidence limits of crocetin for HeLa, A549, and SKOV3 cells were 119.32 μmol/L (104.24–134.83 μmol/L), 101.34 μmol/L (88.07–117.65 μmol/L), and 119.76 μmol/L (97.14–143.40 μmol/L), respectively. Therefore, these results suggest that crocetin effectively suppresses proliferation in the tested cancer cells.

Crocetin inhibits cancer cell proliferation by inducing cell cycle arrest at the G1 checkpoint
To investigate the underlying mechanism for crocetin-induced proliferation inhibition, we examined the cell cycle distribution in crocetin-treated cancer cells using flow cytometric analysis based on cellular DNA content. Crocetin significantly increased the number of HeLa cells in the G1 phase, which was detected at 8 h post crocetin treatment (Figure 2, upper panel). Crocetin-induced G1 phase distribution was time-dependent, and the percentages of cells in the G1 phase increased from 33% in untreated HeLa cells to 41% in HeLa cells after 24 h of crocetin treatment. At the same time, the populations of cells in the S or G2 phases were markedly reduced. Consistent with its effect on HeLa cells, crocetin showed similar effects on the cell cycle of A549 and SKOV3 cells (Figure 2, middle and lower panels). These results indicate that crocetin inhibits cell proliferation by inducing G1 arrest in cancer cells.

Crocetin induces cell cycle arrest through p53-dependent and-independent mechanisms
p53 regulates the G1 checkpoint by activating transcription of genes that influence cell-cycle progression, including cyclin-dependent kinase inhibitor p21WAF1/Cip1 and GADD45[17]. To explore the role of p53 and its downstream genes in crocetin-induced cell cycle regulation, we detected the expression levels of p53 and p21WAF1/Cip1 in crocetin treated cells. As shown in Figure 3A, crocetin induced p53 and p21WAF1/Cip1 accumulation in A549 cells, which have functional wild-type p53. Furthermore, crocetin induced the increase of p53 and p21WAF1/Cip1 in HeLa cells, which express low basal levels of p53 because p53 is degraded via interactions with the E6 gene product of HPV (Figure 3B). Therefore, crocetin may cause G1 arrest through p53 and its downstream p21WAF1/Cip1. Importantly, the p53-independent pathway may also be involved in cell cycle regulation by crocetin, because crocetin also caused G1 arrest in SKOV3 cells that have lost p53 expression and function due to a mutation in the p53 gene (Figure 2, lower panel). Crocetin substantially induced p21WAF1/Cip1 in SKOV3 cells (Figure 3C). These results indicate that a p53-independent mechanism is involved in upregulation of p21WAF1/Cip1, which induces G1 arrest. Altogether, these results suggest that crocetin regulates cancer cell proliferation through p53-dependent and-independent induction of p21WAF1/Cip1.

Crocetin induces cell death by enhancing apoptosis in cancer cells
We further investigated whether crocetin caused cell death in HeLa, A549, and SKOV3 cells after extending the incubation time of drug with the cells. Although marginal cell death was induced by crocetin at early times, significant cell death was detected by the LDH release assay in HeLa cells after crocetin treatment for 72 h (Figure 4A). As shown in Figure 4B, 4C, and 4D, crocetin induced significant cell death in a dose-dependent manner by approximately 40%–50% in all cell lines after treatment with the highest concentration of crocetin. To determine the type of cell death induced by crocetin, HeLa

Figure 1. Crocetin (CRT) inhibits cell proliferation in cancer cells. HeLa (A), A549 (B), and SKOV3 cells (C) were treated with different concentrations of crocetin for 48 h. Cell survival was quantitated using MTT assays. n=3. Mean±SD. *P<0.01 vs control.
cells were incubated with crocetin for different times as indicated. Floating and attached cells were collected, fixed, and stained with PI. The percentage of cells containing sub-G₁ DNA content, which is a measure of apoptosis, was detected using flow cytometric analysis. Figure 5A shows that crocetin treatment resulted in an increase of sub-G₁ phase in a time-dependent manner, which was most evident at 72 h post-treatment. The crocetin-induced apoptosis was further confirmed using flow cytometry after Annexin V-FITC and PI double staining. Early and late apoptotic cells were increased in a dose-dependent manner in crocetin-treated HeLa cells (Figure 5B). The cell distribution in Q1 (Annexin V⁻/PI⁺) in samples that were treated with high concentrations of crocetin was slightly increased. These results indicate necrotic and very late apoptotic cell death (Figure 5B) and suggest that the main killing mechanism of crocetin is inducing apoptosis and possibly necrosis. The decrease of pro-caspase 9, which indicates caspase-9 activation, was detected at 24 h post crocetin treatment. Crocetin treatment also markedly triggered caspase-3 activation and PARP cleavage (115 kDa) to generate a 23-kDa fragment, which are hallmarks of apoptosis induction (Figure 5C). These results demonstrate that crocetin treatment activates the caspase cascade to promote apoptosis. Additionally, the pan-caspase inhibitor Z-VAD-FMK effectively suppressed crocetin-induced cytotoxicity in HeLa, A549, and SKOV3 cells (Figure 5D), substantiating that crocetin induced cell death via apoptosis. Collectively, these results suggest that prolonged exposure of crocetin induces cytotoxicity through apoptosis in cancer cells.

Figure 3. Crocetin induces p53 and p21WAF1/Cip1 in cancer cells. HeLa (A), A549 (B), and SKOV3 cells (C) were treated with crocetin (240 μmol/L) for the indicated time. Using Western blot analysis, p53 and p21WAF1/Cip1 protein expression were evaluated. β-actin was detected as an input control.
cells, A549 cells, and SKOV3 cells with the combination of crocetin and different types of conventional chemotherapeutic drugs. No significant sensitization effects were observed when crocetin was used in combination with cisplatin, carboplatin, etopside, adriamycin, pirarubicin, Taxol, docetaxol, dacarbazine, actinomycin D, or fluorouracil (data not shown). In contrast, simultaneous treatment with crocetin and vincristine synergistically induced cell death (Figure 6A). Notably, the synergistic cytotoxicity was also detected in the vincristine-resistant breast cancer cell line MCF-7/VCR. Although 2 μmol/L vincristine, which is a concentration that kills most of the parental MCF-7 cells, was nontoxic to MCF-7/VCR cells...
DNA, RNA, and protein synthesis in malignant cells\cite{14, 19}. Crocetin inhibits vincristine, an agent as a chemotherapeutic drug or a chemosensitizer for anticancer therapeutics such as vincristine. The mechanism of crocetin remains poorly understood. Crocetin inhibits vincristine-induced cytotoxicity in cancer cells\cite{14}. We demonstrated that crocetin sensitized cancer cells to vincristine sensitivity in the vincristine-resistant breast cancer cell line MCF-7/VCR. To our knowledge, this is the first report showing the chemosensitizing effect of crocetin on vincristine-treated cancer cells because p21\sup{WAF1/Cip1} inhibits the activity of cyclin dependent kinases (Cdks) or proliferating cell nuclear antigen (PCNA). Therefore, p21\sup{WAF1/Cip1} may contribute to G\textsubscript{1} arrest in cancer cells due to the restoration of p53 expression in cisplatin treated HeLa cells\cite{25}. It would be interesting to determine whether crocetin functions to activate p53 through DNA damage. Crocetin-mediated p21\sup{WAF1/Cip1} induction was detected in the p53-null SKOV3 cells, which have rearrangements in the p53 gene that prevent the production of detectable protein products. These results suggest that crocetin activates p21\sup{WAF1/Cip1} through a p53-independent mechanism. Consistent with our results, p53-independent induction of p21\sup{WAF1/Cip1} and concomitant G\textsubscript{1} arrest have been previously reported in malignant cells\cite{26, 27}. The induction of p21\sup{WAF1/Cip1} may contribute to G\textsubscript{1} arrest in crocetin-treated cancer cells because p21\sup{WAF1/Cip1} inhibits the activity of cyclin dependent kinases (Cdks) or proliferating cell nuclear antigen (PCNA). Therefore, p21\sup{WAF1/Cip1} functions as a suppressor of cell cycle progression at the G\textsubscript{1} checkpoint\cite{28, 29}. The roles of cyclins and Cdks in crocetin-induced cell cycle arrest warrant further study.

The MTT assay, which detects viable cells, does not discriminate cell loss that is caused by cell death from that caused by suppression of proliferation. However, the LDH release assay is widely used to detect cell death resulting from apoptosis. Apoptotic cells under tissue culture conditions will eventually leak their cytoplasmic contents because they do not undergo phagocytosis, which occurs in vivo. Using the LDH assay and flow cytometric analysis, we showed that prolonged incubation of crocetin killed cancer cells via apoptosis, which was associated with the activation of caspase-9 to initiate the intrinsic apoptosis pathway. Although the exact mechanism for crocetin-induced apoptotic activation requires further investigation, our results demonstrate that induction of apoptosis at least partly contributes to the anticancer activity of crocetin. In addition, crocetin significantly enhanced the anticancer activity of vincristine in cancer cells. The ability of crocetin to restore vincristine sensitivity in the vincristine-resistant cancer cells suggests a potential role as a chemosensitizer for certain anticancer therapeutics such as vincristine. The mechanism of crocetin to restore vincristine sensitivity in the vincristine-resistant cancer cells suggests a potential role as a chemosensitizer for certain anticancer therapeutics such as vincristine. The mechanism of crocetin to restore vincristine sensitivity in the vincristine-resistant cancer cell lines.
that mediates vincristine-specific sensitization is currently unknown. A vincristine-specific pathway or molecule may be activated in response to crocetin treatment, which enhances the cell killing mechanism of vincristine.

In our study, the IC50 values of crocetin on cell proliferation were from 100–120 µmol/L, which are comparable with that from a study by Dhar[11]. Several animal experiments have shown that high doses of crocetin in vivo are well-tolerated and relatively nontoxic with a potential to exert anticancer activities[11,14,24]. These data indicate the possibility that crocetin may be used at relatively high doses for cancer therapy. The solubility and the bioavailability of crocetin require optimization before being used as an effective anticancer drug. It is also intriguing that the suppression of proliferation and cytotoxic effects of crocetin are not dependent on the p53 status in cancer cells. This advantage is highly relevant because p53 is mutated in approximately 50% of tumors. This study provides novel evidence for the potential use of crocetin as an anticancer agent, which requires further investigation in vivo.

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Author contribution
Ying-jia ZHONG performed the research and analyzed data. Fang SHI and Xue-lian ZHENG assisted with cell culture and cell death experiments. Qiong WANG, Lan YANG, Hong SUN, and Fan HE assisted with flow cytometry and Western blot experiments. Lin ZHANG assisted with data analysis. Yong QIN synthesized and provided crocetin. Xia WANG, Yong LIN, and Lin-chuan LIAO designed the research, analyzed data, wrote and revised the manuscript.

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