RESEARCH COMMUNICATION

Crocin Inhibits Proliferation and Nucleic Acid Synthesis and Induces Apoptosis in the Human Tongue Squamous Cell Carcinoma Cell Line Tca8113

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Abstract

Background: Cancer chemoprevention is a proven effective strategy for oral squamous cell carcinomas (OSCCs). The present study was designed to investigate the effects of crocin, a potential chemopreventive agent, on growth and DNA and RNA content in a human tongue squamous cell carcinoma cell line, Tca8113. Methods: Tca8113 cells were treated with crocin for 24, 48, 72, and 96 h at concentrations of 0.1, 0.2, 0.4, and 0.8 mM. Tumor cell viability was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. In addition, Tca8113 cells were treated with 0.4 mM crocin and cytotoxic effects as an inducer of apoptosis were analyzed using flow cytometry. Furthermore, acridine orange (AO) staining and observation using laser scanning confocal microscopy (LSCM) were used to determine the effects of the drug on nucleic acid synthesis. Results: Crocin decreased Tca8113 cell viability and growth remarkably at 24, 48, 72, and 96 h, in a concentration-dependent manner ($P<0.05$). In addition, 0.4 mM crocin significantly induced both early and late apoptosis of Tca8113 cells. Moreover, the cellular DNA and RNA content was significantly downregulated by 0.4 mM crocin compared with the negative control ($P<0.01$). Conclusions: Our observations support the feasibility of applying crocin as a chemoprophylactic agent and treatment for OSCCs.

Keywords: Crocin - oral squamous cell carcinoma - cell viability - nucleic acid synthesis

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies, accounting for more than 80% of malignancies of the oral cavity and 3–5% of total malignancies (Rafael et al., 1999). Recently, the prevalence of OSCC has risen in most countries worldwide (Jemal et al., 2004). Although OSCC is characterized by easy shift and poor prognosis, even with surgery or other systemic therapies, its cancer incidence and recurrence rate remain high. Thus, the importance of prevention must be highlighted.

Chemoprevention is one extremely promising strategy for cancer prevention that was introduced by Michael Sporn in 1976. It refers to the use of natural, synthetic, or biological agents, alone or in combination, to reverse or prevent the development of cancers (Lee, 1985). Chemoprevention of OSCC is based on two effective strategies: primary and secondary prevention. In addition, chemopreventive agents can be used to prevent further conversion of premalignant mucosa lesions, to reduce the rate of second primary tumors.

Several improved chemopreventive compounds have been developed against oral cancers, including retinoids, beta-carotene, vitamin E, selenium, nonsteroidal anti-inflammatory agents, and natural agents. Retinoids (i.e., vitamin A and its biologically active derivatives) are the prime candidates as chemoprevention substances and are used widely to reverse the development of precancerous oral lesions, as they stimulate cell proliferation, induce apoptosis, strengthen immunity, and reduce mucosa dysplasia. However, although a number of clinical trials confirmed their effective role in tumor inhibition, they also revealed their limitations (Waun, 1986; Okuno et al., 2004). First, lesions may recur on stoppage. Second, side effects happen occasionally. Patients with mild lesions may experience dry mouth or lips, facial erythema, and cheilitis, whereas more severe cases may develop hypertriglyceridemia, renal damage, premature beat, and teratogenesis. Thus, we are searching for more powerful

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alternative compounds with structural similarities to retinoids, but lower or no toxicity.

As drugs is needed for long-term preventive efficacy in prevention of oral cancer and the numbers of patients try herbal medicines has ever been on the rise (Kaefer and Milner, 2008), we turned to Chinese medicine. Research exploring new botanical candidates with potential anticancer effects was imperative and allowed the development of safe and efficacious chemoprevention. *Crocus sativus*, known commonly as saffron, is cultivated widely in Iran and other countries. Modern pharmacological studies demonstrated that saffron, or its active constituents, has anticonvulsant, antidepressant, anti-inflammatory, antioxidative, and antitumor activities (Abdullaev, 2002; Abdullaev and Espinosa-Aguirre, 2004; Hosseinzadeh and Younesi, 2002; Hosseinzadeh and Karimi, 2004). Furthermore, several previous studies suggest that saffron possesses antitumor and anticarcinogenic activities and has no cytotoxic effects in nonmalignant cells.

Several important components of saffron, e.g., safranal, picrocrocin, crocetin, and crocin, are proven pharmacologically active compounds (Salomi and Nair, 1991). In addition, most of these chemical compounds are structurally similar to retinoids. As the main constituent of Crocus sativus extract, crocin is considered as the most effective of its components. Several studies have documented that crocin inhibits the growth of many malignant cancer cells, such as HeLa cells, acute leukemia cells, and non-small cell lung cancer cells (Tarantilis et al., 1994; Escribano et al., 1996).

However, to the best of our knowledge, no scientific reports are available on the inhibitory effect of crocin on OSCC. The present study was designed to investigate the effects of crocin on the viability and DNA and RNA content of Tca8113 cells.

Materials and Methods

Cell culture

The human tongue squamous cell carcinoma cell line Tca8113 (purchased from the Chinese Academy of Sciences) was cultured in RMPI 1640 medium (Gibco, Grand Island, NY, USA) with 10% fetal calf serum (Gibco) and 100 U/mL penicillin and 100 Ag/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2 in an incubator (SANYO, Japan). The medium was replaced every 2–3 days and cells were digested using trypsin and subcultured in a new plate when they reached ~80% confluence.

Crocin separation and purification

Dried saffron stigmas (Iran; purchased from the Chengdu medicine market) were stored in the dark at 4°C in a dry place. Crocin was extracted and purified by Professor Zhang at the Natural Plant Application Laboratory, Sichuan Normal University (Hong, 2004).

A UV/Vis spectrophotometer was used (UV-1700 (E) 230CE, Shimadzu, Japan) to assess the purity of the prepared crocin (scan range, 200–600 nm). After successful appraisal, solutions with different concentrations of crocin (0.1, 0.2, 0.4, and 0.8 mM) were prepared.

Light microscopy observation

Tca8113 cells were seeded at a concentration of 5×10^4/mL in a 24-well plate until 80% confluence was reached, and were then incubated with 0.4 mM crocin for 48 h. After conventional Giemsa staining, cell morphology was examined using an inverted phase contrast microscope (IX70, Olympus, Japan). Permanent slides were prepared for analysis.

MTT assay

Cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Briefly, cells were added to a 96-well culture plate at a concentration of 5×10^4/mL. Cells were treated with serial concentrations of crocin (0.1, 0.2, 0.4, and 0.8 mM) for 24, 48, 72, and 96 h. Untreated cells were set as the control group. Subsequently, all cells were incubated with MTT (Sigma, St. Louis, MO, USA) solution (5 mg/mL) for 4 h at 37°C. The medium was removed and formazan was solubilized with DMSO (200 μL/well). The absorption was measured at 570 nm using an HTS. 7000 Plus Bio Assay Reader (Perkin Elmer, Waltham, MA, USA). Growth inhibition was calculated using the following equation: $IR = (1 - A_{sample} / A_0) \times 100\%$, where A_{sample} refers to the absorbance of the sample in the presence of crocin and carboplatin and A_0 is the absorbance of the control in the absence of samples. The experiment was performed in triplicate and the results were expressed as mean values.

Annexin V–FITC/PI stain and flow cytometry

After treatment with 0.4 mM crocin for 24 h, 5×10^5 adherent cells were trypsinized and incubated with FITC-conjugated annexin V and propidium iodide (PI) for 10 min in the dark. Apoptosis arrest was determined using an Annexin V–FITC and PI Apoptosis Detection Kit (BioVision, USA), according to manufacturer's instructions. Assessment of samples was accomplished using a flow cytometer (Epics Elite ESP; Coulter, USA). Cells in the FITC-positive and PI-negative fractions were defined as apoptotic cells. Cells in the FITC-positive and PI-positive fractions were defined as late apoptotic/necrotic cells.

Fluorescent microscopic analysis of DNA and RNA synthesis using AO staining

Tca8113 cells were seeded in a 24-well plate at a concentration of 5×10^4/mL. The cells were treated with 0.4 mM crocin for 48 h at 37°C. After aspiration of the medium, the cells were washed with Hank's buffer for 3–5 s, placed in 95% ethanol for 10–15 min, and subjected to 1% acetic acid acidification for 30 s. Cells were then washed with PBS buffer (pH 4.5–5.0) three times, stained with 0.01% acridine orange (AO) (Sigma) for 15 min, and washed with PBS buffer for 1 min. After adding 0.1 M $CaCl_2$ for 30 s, cells were finally washed with PBS buffer. The stained cells were examined using a fluorescence microscope (Leica TCS SP2, Germany) at 488 nm excitation.

We selected five fields randomly in each view, and each

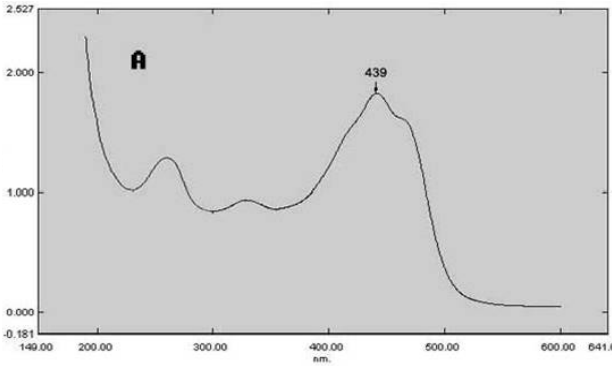

Figure 1. Spectral Analysis of Crocin. The maximum absorption appears near 439 nm. The scan range is from 200 to 600 nm

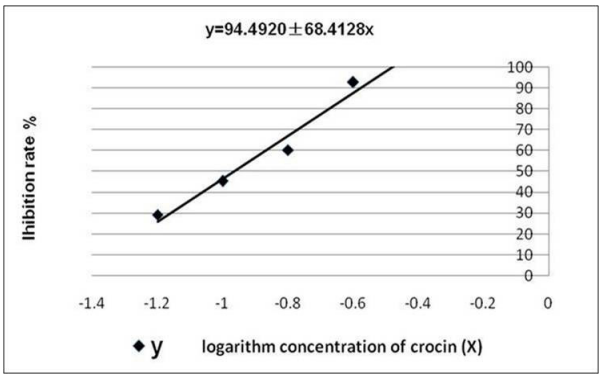

Figure 3. Dose-inhibition Curve of Crocin in Tc8113 Cells after 48 h. After treatment with crocin for 48 h, the IC50 is ~0.2237 mM

field contained four cells, accounting for 20 cells in total. Green fluorescence referred to the cellular DNA, whereas red fluorescence represented cellular RNA. The intensity and extent of the fluorescent signals corresponded to the cellular DNA and RNA content. The results were expressed as mean values.

Statistical analysis

Experimental data were expressed as mean ± standard deviation (x ± s). Statistical analyses were performed using SPSS 13.0. The differences between two groups were analyzed using one-way analysis of variance (ANOVA). In linear regression analyses, the regression coefficient was tested by ANOVA. For all tests, significance was set at $P<0.05$.

Results

Crocin separation and purification

Chromatography (Figure 1) showed that crocin was the major compound in the extract of saffron, with maximum absorption near 439 nm. The purity of crocin was over 96%.

Morphological changes in Tca8113 cells treated with crocin

Morphological changes in cells treated with crocin were observed using inverted light microscopy. There was no significant difference between the crocin group and the control group during the first 24 h. However, after another 24 h, the untreated cells grew compact and smooth, whereas cells treated with crocin exhibited typical features

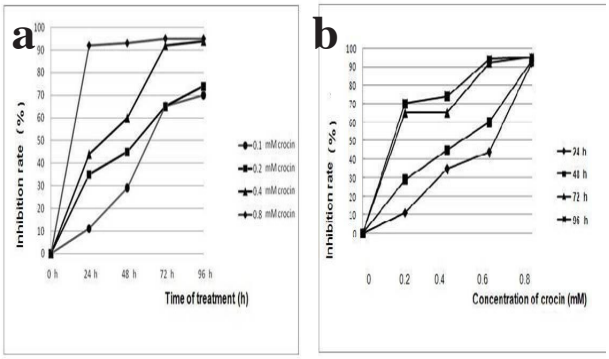

Figure 2. Tca8113 Cells were Treated with Different Concentrations of Crocin for 24, 48, 72, and 96 h. Cell viability was quantitated using an MTT assay. a) Time-inhibition curve of crocin shows that, at equal concentration, crocin enhances the suppression effect on Tca8113 cells with time; b) Dose-inhibition curve of different concentrations of crocin in Tca8113 cells shows that, at the same time point, higher concentrations of crocin inhibit more Tca8113 cells

of apoptosis, such as star-like morphology, cell shrinkage, reduced cell wall adherence, and pyknotic nuclei.

Growth inhibitory effects of crocin on Tca8113 cells

Tca8113 cells were treated with crocin at various concentrations (0.1, 0.2, 0.4, and 0.8 mM) for 96 h and the viability of the cells was determined using an MTT assay at the 24, 48, 72, and 96 h time points. Results showed that the viability of Tca8113 cells treated with crocin at varying concentrations displayed a significant decrease compared with that of untreated cells ($P<0.05$). Crocin exhibited a significant cell viability inhibitory impact on Tca8113 cells, both in a dose- and time-dependent manner (Figure 2). At 48 h, the inhibition rate was based on the linear trend observed in Figure 3. The IC50 was 0.2237 mM.

Cytotoxic effect of crocin as an inducer of apoptosis

The Tca8113 cells visualized fluorescently were treated with crocin and analyzed using flow cytometry. An apoptosis rate of 2.8% was observed in Tca8113 cells after treatment with 0.4 mM crocin for 24 h, whereas an apoptosis rate of 0.5% was observed for the control sample. A late apoptosis/necrosis rate of 14.53% was observed for Tca8113 cells after treatment with crocin, whereas a late apoptosis/necrosis rate of 4.73% was observed for the control.

In the gated areas, the upper right panel displays the population of late apoptotic cells and the lower right panel displays the early apoptotic cells with increased annexin V staining (Figure 4). These results indicate clearly that treatment with crocin significantly induces both early and late apoptosis in Tca8113 cells.

Inhibitory effects of crocin on cellular DNA and RNA synthesis

Significant differences in the recorded values of DNA and RNA fluorescence intensity and in the ratio of RNA/DNA were found between the two groups ($P<0.01$). These results suggest that crocin inhibits the synthesis of cellular nucleic acids (Figures 5 and 6).

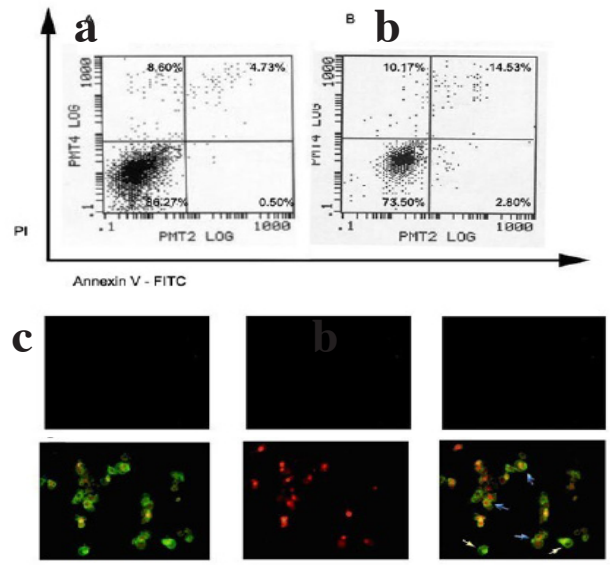

Figure 4. Flow Cytometry Analysis of the Apoptosis Induced in Tca8113 Cells Treated with Crocin, as Assessed Using Annexin. FITC/PI expression analysis. a) Untreated Tca8113 cells. b) Cells were treated with 0.4 mM crocin and then collected for staining with FITC-conjugated annexin V. c) Untreated cells were set as the control group (upper) and cells (0.4 mM crocin group) stained with annexin , FITC (green fluorescence) and PI (red fluorescence) and observed using fluorescence microscopy (lower)

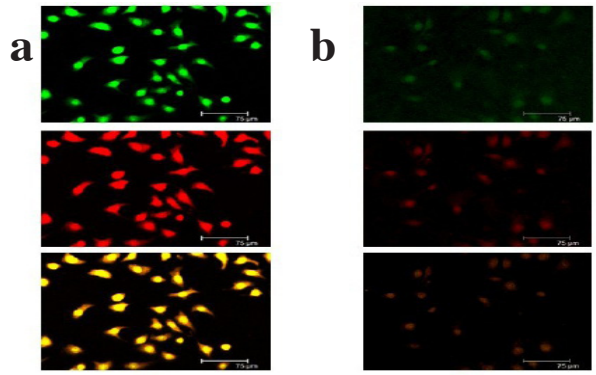

Figure 5. Cellular DNA and RNA Changes in Tca8113 Cells Treated with Crocin at a Concentration of 0.4 Mm for 48 h. a) Negative control Tca8113 cells. b) Crocin-treated cells (0.4 mM). Green fluorescence represents cellular DNA content, whereas red fluorescence represents cellular RNA content. Yellow fluorescence represents both DNA and RNA. AO staining: observation using Laser Scanning Confocal Microscopy. Magnification, 200×

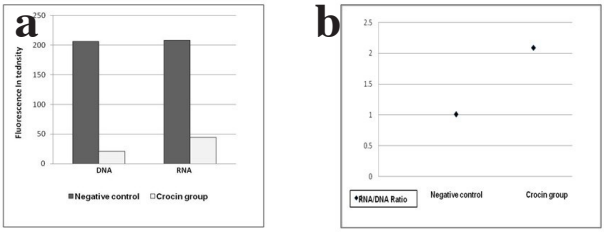

Figure 6. Crocin Inhibits the Synthesis of Cellular Nucleic Acids. a) Significant downregulation of the DNA and RNA content was observed in the crocin group compared with the negative control group ($P<0.01$). b)The ratio of RNA/DNA in the crocin group was higher than that of the control counterpart

Discussion

Chinese medicine, an intensive research area for chemopreventive drugs, has been used in the last decades to prevent deterioration in precancerous diseases. Crocin, a potential candidate, is known for its anticancer properties. In this study, we successfully isolated high-purity crocin from saffron and demonstrated its antitumor effects on Tca8113 cells. To the best of our knowledge, this is the first suggestion of crocin-induced inhibitory effects on OSCCs.

In vitro cell morphology observation and a proliferation inhibition test using an MTT viability assay confirmed that crocin had cytotoxic activity against the Tca8113 cell line. Using light microscopy, we showed that Tca8113 cells treated with crocin exhibited obvious morphological changes and altered growth patterns. The morphological changes observed reflected profound metabolic alterations, including changes in nucleic acid synthesis. The inhibitory effects of crocin on cell growth in terms of dose-dependent cytotoxicity or the induction of apoptosis were also assessed. We established an IC50 value of 0.2237 mM, which was consistent with the results of previous investigations. The observed IC50 values for crocin ranged from 0.4 to 3 mM for epithelium-derived malignancies, whereas this value generally ranged from 0.6 to 2 μM for several mesenchymal tumors (Abdullaev and Espinosa-Aguirre, 2004), such as the HL60, K562, and L1210 cell lines, which suggests that mesenchymal tumors are more sensitive to crocin.

However, the mechanism underlying the antitumor effects of crocin remains unclear. Different hypotheses regarding the mode of the anticarcinogenic and antitumor actions of crocin have been proposed. Escribano et al. (1996) found crocin induced apoptosis on HeLa and K562 cells. And other studies showed that free radical chain reaction played role to explain its anti-tumor behavior (Premkumar et al., 2001). In addition, inhibition on nucleic acid synthesis, the antagonistic activity of 7,12-dimethylbenz(α)anthracene (DMBA), immunomodulatory ability and hormonal factors may also be considered several potential mechanisms (Nair, 1995; Escribano, 1999). But in our study, we focused on cellular DNA and RNA synthesis Using Laser Scanning Confocal Microscopy(LSCM), we demonstrated that 0.4 mM crocin had inhibitory effects on cellular DNA and RNA synthesis in Tca8113 cells. Regarding the magnitude of this inhibition, the content of DNA decreased more than that of RNA. We hypothesize that crocin induces DNA damage first and that, because of template deficiency, the synthesis of RNA is affected gradually.

In summary, crocin inhibited the viability of the human tongue squamous cell carcinoma cell line Tca8113 in vitro, which was accompanied by downregulation of the synthesis of DNA and RNA. The results of this study provide clues for the investigation of the biochemical mechanisms of the antitumor effects of crocin in Tca8113 cells and may represent valuable information for the development of drugs for controlling the progression of OSCC. Further in vitro and in vivo work is required to clarify the action of crocin in various tumors and identify the specific molecular mechanisms involved.

Acknowledgements

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