NRC Research Press

ARTICLE

Proposed cytotoxic mechanisms of the saffron carotenoids crocin and crocetin on cancer cell lines

Se Hyeuk Kim, Jung Min Lee, Sun Chang Kim, Chan Bae Park, and Pyung Cheon Lee

Abstract: We investigated the cytotoxic activities of crocin and crocetin, 2 major carotenoids isolated from the stigma of *Crocus sativus* (saffron), on 5 human cancer cell lines and proposed their possible anticancer mechanisms. Crocetin, a glycosylated carotenoid, showed approximately 5- to 18-fold higher cytotoxicity than crocin, a carboxylic carotenoid (IC$_{50}$ of 0.16–0.61 mmol/L for crocetin vs. 2.0–5.5 mmol/L for crocin). This suggests that structural differences account for the different efficacies between them. Fluorescence-activated cell sorting (FACS) analysis showed that crocetin induced a significant level of cellular reactive oxygen species (ROS) in HeLa cells, whereas crocin did not. This ROS induction supported the cytotoxicity of crocetin, but not of crocin. A significant activation of nuclear factor erythroid 2-related factor 2 (Nrf2) was observed in both HeLa cells treated with crocin and crocetin: a 3.0-fold increase by 1 mmol/L crocetin and a 1.6-fold increase by 0.8 mmol/L crocin compared to the control. Furthermore, both crocetin and crocin reduced the protein expression of lactate dehydrogenase A (LDHA), one of the targets for chemoprevention in cancer cells, by 34.2% and 10.5%, respectively, compared to the control in HeLa cells. These findings suggest that crocetin and crocin have different mechanisms for their observed cytotoxicity in cancer cell lines.

Key words: crocin, crocetin, ROS, LDHA.

Résumé : Nous avons examiné l'activité cytotoxique de deux caroténoïdes importants, l'un glycosylé (crocine) et l'autre, carboxylé (crocétine), isolés du stigmate de *Crocus sativus,* sur 5 lignées cellulaires cancéreuses humaines, et nous avons proposé de possibles mécanismes d'action anticancéreuse. La crocétine était environ de 5 à 18 fois plus cytotoxique que la crocine (CI$_{50}$ de 0.16 – 0.61 mmol/L pour la crocétine comparativement à 2.0 à 5.5 mmol/L pour la crocine), suggèrant que les différences de structures sont responsables des différences d'efficacité. L'analyse par FACS a montré que la crocétine induisait un niveau significatif d'espèces réactives d'oxygène (ERO) cellulaires chez les cellules HeLa, contrairement à la crocine. Cette induction d'ERO soutenait la cytotoxicité de la crocétine, contrairement à la crocine. Une activation significative de facteur nucléaire Nrf2 (*nuclear factor E2-related factor 2*) a été observée chez les cellules HeLa traitées à la crocine et la crocétine : une augmentation de 3 fois par 1 mmol/L de crocétine et de 1.6 fois par 0.8 mmol/L de crocine, comparativement au contrôle. De plus, tant la crocétine que la crocine réduisaient l'expression de la lactate déshydrogénase A (LDHA), une des cibles de la chimioprévention du cancer, de 34.2 % et 10.5 % respectivement, comparativement au contrôle chez les cellules HeLa. Ces données suggèrent de manière irréfutable que la crocétine et la crocine exercent une activité cytotoxique envers les cellules cancéreuses par l'intermédiaire de différents mécanismes. [Traduit par la Rédaction]

Mots-clés : crocine, crocétine, ERO, LDHA.

Introduction

Carotenoids are a class of isoprenoid derivatives and more than 700 carotenoids have been identified to date(Lee et al. 2003). They are biosynthesized by microorganisms, fungi, algae, and plants for photoprotection as scavengers of reactive oxygen species (ROS), for electron transfer as photosynthetic pigments and for regulation of membrane rigidity (Holt et al. 2005). Animals consume carotenoids from diets of fruits and vegetables; the oxidative cleavage of consumed carotenoids (including xanthins) is followed for retinal formation and transcriptional system activation (Sharoni et al. 2012). In addition, cleavage products named apocarotenoids are known to be biologically active as anticancer agents and cellular modulators, including the retinoic acid receptor retinoid X receptor (RXR), peroxisome proliferator-activated receptor (PPAR), and estrogen receptor (Sharoni et al. 2012).

Among the apocarotenoids of biotechnological significance, saffron, the dried dark-red stigma from *Crocus sativus* native to Southwest Asia, has been used traditionally to treat some diseases and as flavoring and coloring for foods since ancient times (Gutheil et al. 2012). Crocin is a major glycosylated carotenoid found in saffron; crocetin, consisting of carboxyl groups at both ends of the backbone, is a carboxylic carotenoid (Fig. 1a). Recently, it has been reported that the carotenoids in saffron induce apoptosis and inhibit proliferation and synthesis of DNA, RNA, and proteins in various types of cancer cells (Tavakkol-Afshari et al. 2008; Aung et al. 2007; Kanakis et al. 2007a, 2007b; Escribano et al. 1996). Among the cancer cell lines, HeLa is one of the most widely studied (Gutheil et al. 2012). However, details on how to induce apoptosis and inhibit proliferation of the cancer cell are limited at present.

S.H. Kim and P.C. Lee. Department of Molecular Science and Technology and Department of Applied Chemistry and Biological Engineering, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 443-749, Republic of Korea.
J.M. Lee and C.B. Park. Department of Physiology, Ajou University School of Medicine, Woncheon-dong, Yeongtong-gu, Suwon 443-721, Republic of Korea.
S.C. Kim. Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1 Gwanhangno, Yusong-gu Taejon 305-701, Korea.

Corresponding authors: Pyung Cheon Lee (e-mail: pclee@ajou.ac.kr) and Chan Bae Park (e-mail: chanbaepark@ajou.ac.kr).

Fig. 1. *a*) Chemical structures of crocin and crocetin and *b*) viabilities of 5 cancer cell lines treated with varying concentrations of crocin and crocetin. Cells (2500 cells/well) were treated with varying concentrations of crocin or crocetin, after which the viability of the treated cells was measured with an MTT assay and normalized with control cells. Values (means ± SD) are expressed relative to the control cells and each experiment was performed in triplicate (*$p < 0.05$, **$p < 0.001$). ●: crocin; ○: crocetin.
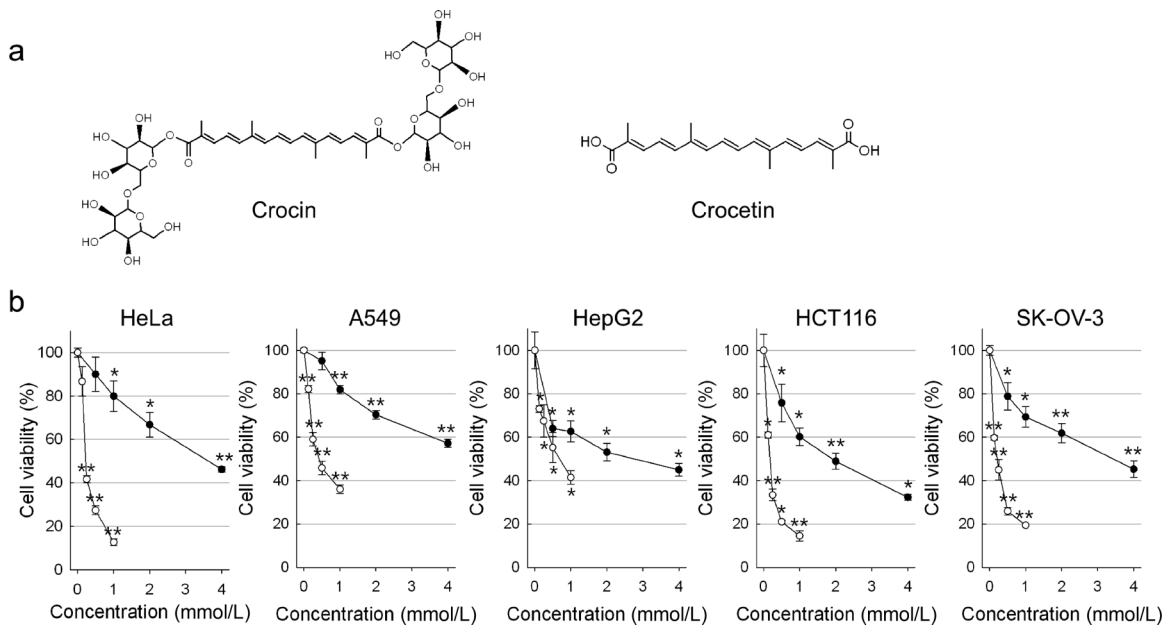
Phytochemicals, such as terpenes and phenolics, are known to activate nuclear factor erythroid 2-related factor 2 (Nrf2), and are one of the most important factors in cancer chemoprevention (Surh 2003). Nrf2 is a transcription factor that induces antioxidant response element (ARE) transcription, such as NAD(P)H:quinone oxidoreductase-1 (NQO1), NAD(P)H:quinone oxidoreductase-2 (NQO2), glutathione S-transferases, and heme oxigenase-1 (HO-1). Although Nrf2 is suppressed by Kelch-like ECH-associated protein 1 in a cytosol under normal conditions, Nrf2 is released and translocated to the nucleus for the transcriptional induction of ARE upon oxidative or electrophilic stress (Itoh et al. 1997).

Recently, lactate dehydrogenase A (LDHA), the predominant form of 5 isozymes of LDH, has received attention as a target of cancer suppression (Zhou et al. 2010; Fantin et al. 2006). The underlying mechanism is that inhibition of LDHA activity decreases the conversion reaction rate of pyruvate to lactate, decreasing mitochondrial membrane potentials and cellular ATP levels, and finally inducing oxidative stress and apoptosis in cancer cells (Fantin et al. 2006). This serves to elucidate or propose mechanisms for the cytotoxicity of bioactive compounds in cancer cell lines.

In this study, we examined the cytotoxic effects of crocin and crocetin, 2 major carotenoids in saffron, on 5 cancer cell lines, and then propose possible mechanisms of actions for the 2 compounds on these cell lines. We found that crocetin induced a significant level of ROS, whereas crocin did not. However, the activation of Nrf2 and inhibition of LDHA expression were observed in both HeLa cells treated with crocin and crocetin. Therefore, crocetin and crocin exert their cytotoxic effects through different mechanisms despite their structural similarities.

Materials and methods

Reagents

Crocin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), *N*-acetylcysteine (NAC), sodium deoxycholate, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Nonidet P-40 (NP-40) was purchased from Generay Biotech (Shanghai, China). Crocetin was purchased from MP Biomedicals

(Solon, OH, USA). The fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) was purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). Primary antibodies against NQO1, HO-1, LDH, Lamin-A, and β-actin were purchased from Abcam (Cambridge, UK).

Cell culture

The 5 human cell lines, A549, HepG2, HCT-116, SK-OV-3, and HeLa cells, were cultured in DMEM supplemented with 5% heat-inactivated FBS. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell viability – MTT assay

Cell viability was determined with an MTT assay. Cells were seeded (2500 cells/well) onto 96-well cell culture plates and allowed to grow overnight. Crocin and crocetin (in DMSO) were dissolved in DMEM supplemented with 5% FBS using serial dilution. A 1/500 volume of DMSO was used as the vehicle control for crocetin. 5 mmol/L or 7 mmol/L NAC were co-treated with crocin or crocetin. The MTT solution was prepared by dissolving MTT in Dulbecco's phosphate buffered saline (DPBS, 5 mg/mL) and diluted in DMEM supplemented with 5% FBS to a final concentration of 1 mg/mL in the assay. After 48 h of treatment, cells were washed with DPBS twice and further incubated in fresh MTT solution for 4 h at 37 °C. After removing the MTT solution, formazan in DMSO was added (100 μL/well) and the absorbance was recorded at 575 nm with a plate reader (Bio-Rad, Hercules, CA, USA).

Western blot analysis

HeLa cells were seeded (2×10^5 cells/well) onto 6-well cell culture plates. After 24 h of treatment, cells were washed twice with DPBS and lysed with radioimmunoprecipitation assay (RIPA) buffer (150 μL/well) supplemented with protease inhibitor cocktail and 1 mmol/L NaF. Following centrifugation (12 000*g*, 4 °C) for 1 h, the supernatant was transferred and quantified by Bradford assay. Equal amounts of proteins were mixed with 6× sample buffer and boiled for 5 min. Proteins were electrophoretically separated on a 12% polyacrylamide gel and transferred to a nylon membrane for

2 h. The blot was blocked with Tris-buffered saline with Tween 20 (TBST) containing 3% (*w/v*) BSA at 4 °C overnight. After washing 3 times with TBST, the blot was incubated for 1 h with TBST containing the indicated primary antibody and 1% BSA. The membrane was washed 3 times with TBST and incubated for 1 h with TBST containing horseradish peroxidase–conjugated secondary antibody and 1% BSA. After washing 3 times with TBST, enhanced chemiluminescence (ECL) substrate solution (Bio-Rad, Hercules, CA, USA) was added dropwise onto the blot and visualized on X-ray film.

Quantitative real-time PCR

HeLa cells were seeded (2×10^5 cells/well) onto 6-well cell culture plates. After 24 h of treatment, cells were washed twice with DPBS and total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany). 1 μg of total RNA was used for cDNA synthesis with random primers by using a High-Capacity cDNA Reverse Transcription kit (Applied Bioscience, Foster City, CA, USA). The cDNA levels were analyzed using a Rotor-Gene Q (Qiagen, Hilden, Germany) with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Each sample was measured in triplicate in a reaction mixture of 20 μL containing 200 nmol/L of primers. Primers for the specific amplification of HO-1, NQO1, NQO2, and β-actin were as follows: HO-1 sense, CAGGCAGAGAATGCTGAGTTC; HO-1 antisense, GATGTTGAGCAGGAACGCAGT; NQO1 sense, ATGGTCGGCA-GAAGAGCACTGATCG; NQO1 antisense, TTTTCTAGCTTTGATCTGGTT-GTCAGTTGGG; NQO2 sense, CCACGAAGCCTACAAGCAAAG; NQO2 antisense, CCAGTACAGCGGGAACTGAAATA; β-actin sense, GCGGG-AAATCGTGCGTGACATT; β-actin antisense, GATGGAGTTGAAGG-TAGTTTCGTG. Primers were purchased from Macrogen (Seoul, Korea).

Reporter assay

HeLa cells were seeded (2.5×10^4 cells/well) onto 24-well cell culture plates and were transfected with pARE-luc, which possess a *cis*-regulatory DNA sequence located in the promoter of ARE element enzymes, for 24 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Additional pRL-CMV was co-transfected for normalization of luciferase signals on various cell numbers. Crocin or crocetin were treated on the reporter-transfected HeLa cells for 6 h and monitored by fluorospectrometer using Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) according to manufacturer's protocol. Each experiment was performed independently and normalized to the Renilla luciferase signal.

Detection of ROS

Cells were seeded (1.5×10^5 cells/well) onto 12-well cell culture plates and treated with 4 mmol/L crocin or 1 mmol/L crocetin for 24 or 48 h. After treatment, cells were washed twice with DPBS and detached by trypsin. Cells were harvested and trypsin was inactivated after washing with DMEM supplemented with 5% FBS. Cells were incubated for 20 min at 37 °C in 5 μmol/L of DCF-DA (in PBS) and washed with DPBS. DCF was monitored by flow cytometer (BD Biosciences, San Jose, CA, USA). Histograms were overlaid using WinMDI program.

LDH activity assay

HeLa cells treated with crocin/crocetin on 12-well cell culture plates (2×10^5 cells/well) for 24 h were suspended with 300 μL lysis/assay buffer (50 mmol/L Tris–Cl pH 7.4, 150 mmol/L NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, proteinase inhibitor cocktail). Lysis was performed by passing suspended cells through a 1 mL syringe 3 times then the supernatants were pooled and quantified using a RC DC protein assay kit (Bio-Rad, Hercules, CA, USA) after centrifugation at 4 °C (15 700*g*) for 15 min, LDHA activity was monitored by measuring UV absorbance at 340 nm at 25 °C with 1 mmol/L pyruvate and 0.5 mmol/L NADH in 100 mmol/L potassium phosphate buffer (pH 7.4) using a Spectramax Plus384 spectropho-

Table 1. IC$_{50}$ values of crocin and crocetin on the human cancer cell lines.

Carotenoids	Cell lines				
	A549	HepG2	HCT-116	HeLa	SK-OV-3
Crocin (mmol/L)	5.48	2.87	1.99	3.58	3.35
Crocetin (mmol/L)	0.41	0.61	0.16	0.22	0.19

tometer (Molecular Devices, Sunnyvale, CA, USA). Experiments were performed 3 times independently and results were normalized to the protein concentrations.

Statistical analysis

Values were represented as the mean ± standard deviation (SD) from 3 independent experiments. The IC$_{50}$ of crocin/crocetin on 5 cancer cell lines were calculated from equations derived from dynamic fit curves of each inhibition curve using SigmaPlot (Systat Software, San Jose, CA). Comparisons between groups were performed by one-way ANOVA with Bonferroni test model; $p < 0.05$ and $p < 0.001$ were used for statistical significance.

Results

Cytotoxicity of crocin and crocetin on 5 human cancer cell lines

Crocin and crocetin were applied at varying concentrations to 5 cancer cell lines (adenocarcinomic alveolar basal cell line, A549; hepatocellular liver cell line, HepG2; colorectal colon epithelial cell line, HCT-116; adenocarcinomic cervical epithelial cancer cell line, HeLa; and adenocarcinomic ovarian epithelial cell line, SK-OV-3). MTT assays revealed that both crocin and crocetin decreased the viability of all 5 cell lines even though the IC$_{50}$ of crocin and crocetin was different depending on the cell line (Table 1). The viability of the cell lines, treated with crocetin or crocin at varying concentrations, decreased in a dose-dependent manner as for the HeLa cells (Fig. 1b). However, the extent of the viability of all cell lines significantly varied between crocetin and crocin: crocetin was more effective by 5–18 times than crocin in the cytotoxicity of cell lines. More than 85% of human fibroblast cells were viable when 4 mmol/L of crocin or 1.0 mmol/L of crocetin was treated (data not shown).

Intracellular ROS levels in crocin/crocetin-treated HeLa cells

We chose HeLa cells as a model cell line to obtain supporting evidence for the cytotoxicity mechanism of action for crocin or crocetin on the cancer cell lines examined and measured intracellular ROS levels in NAC-treated and nontreated HeLa cells by flow cytometry. After verifying NAC concentration ranges in which no cytotoxicity effects on HeLa cells were observed, 5.0 mmol/L or 7.0 mmol/L NAC were co-treated with crocin or crocetin. A higher level of ROS was detected in only HeLa cells with crocetin after both 24 and 48 h of treatment compared to both control and crocin-treated cells (Fig. 2a). The ROS levels decreased in a dose-dependent manner by adding NAC (5.0 and 7.0 mmol/L) onto the HeLa cells treated with crocetin, supporting the fact that crocetin accounts for the induction of the observed intracellular ROS in HeLa cells (Fig. 2a). Unlike the significant induction of ROS by crocetin, no detectable ROS was observed in crocin-treated HeLa cells after 24 and 48 h of treatment.

ROS-associated cytotoxicity of crocin/crocetin

The elevated ROS-associated cytotoxicity was then investigated using an MTT assay following the addition of crocin, crocetin, crocin + NAC, or crocetin + NAC to HeLa cells. HeLa cells treated with 1 mmol/L crocetin restored their viability up to 83.5% ± 7.3% ($p < 0.05$) from 43% ± 3.4% ($p < 0.05$) when 7.0 mmol/L NAC was added (Fig. 2b). However, HeLa cells treated with 4.0 mmol/L crocin did not significantly restore their viability when NAC

Fig. 2. ROS generation in crocin/crocetin-treated HeLa cells. *a*) Flow cytometry histogram of HeLa cells treated with 4 mmol/L crocin or 1 mmol/L crocetin for the indicated treatment times. 5 mmol/L or 7 mmol/L of NAC was used as an antioxidant. ROS production was monitored after staining the cells with 5 µmol/L DCF-DA for 20 min at 37 °C. Histograms were overlaid using WinMDI program. (*b*) Effects of the co-treatment of antioxidant NAC with 4 mmol/L crocin or 1 mmol/L crocetin on HeLa cell viability. Cells (1.5×10^5 cells/well) were co-treated with 5 mmol/L or 7 mmol/L NAC for 48 h. Cell viability was measured with an MTT assay. Values are expressed relative to control cells and are the means ± SD of 3 independent experiments, each performed in triplicate, (*$p < 0.05$, **$p < 0.001$).
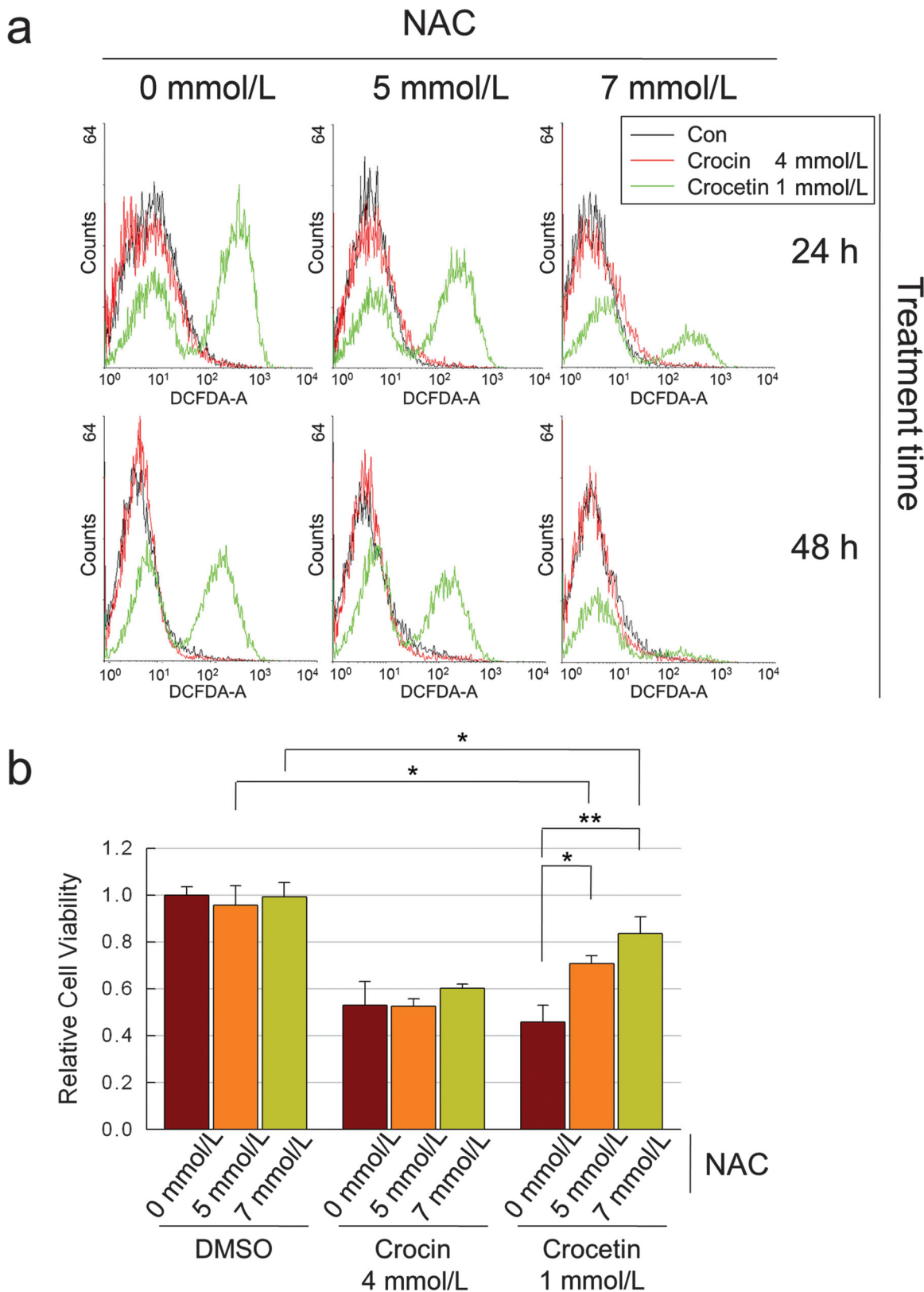
Fig. 3. Activation of Nrf2 and its target genes induced by crocin or crocetin in HeLa cells. *a*) HeLa cells were treated with crocin (32 and 800 μmol/L) or crocetin (31 and 1000 μmol/L) as well as tBHQ (50 μmol/L) as a positive control. Values are expressed as the fold increase in luciferase activity, relative to the activity of the control cells and are the means ± SD of 3 experiments, each performed in triplicate, (*$p < 0.05$, **$p < 0.001$). (*b*) HeLa cells were treated with 4 mmol/L crocin or 1 mmol/L crocetin for 24 h, and total RNA was prepared. mRNA levels of HO-1, NQO1, and NQO2 were analyzed by RT–PCR as described in the Materials and Methods. NT: no treatment. (*c*) Protein expression levels of NQO1 and HO-1 were analyzed by Western blot analysis. β-actin was a control target gene to ensure equal loading.
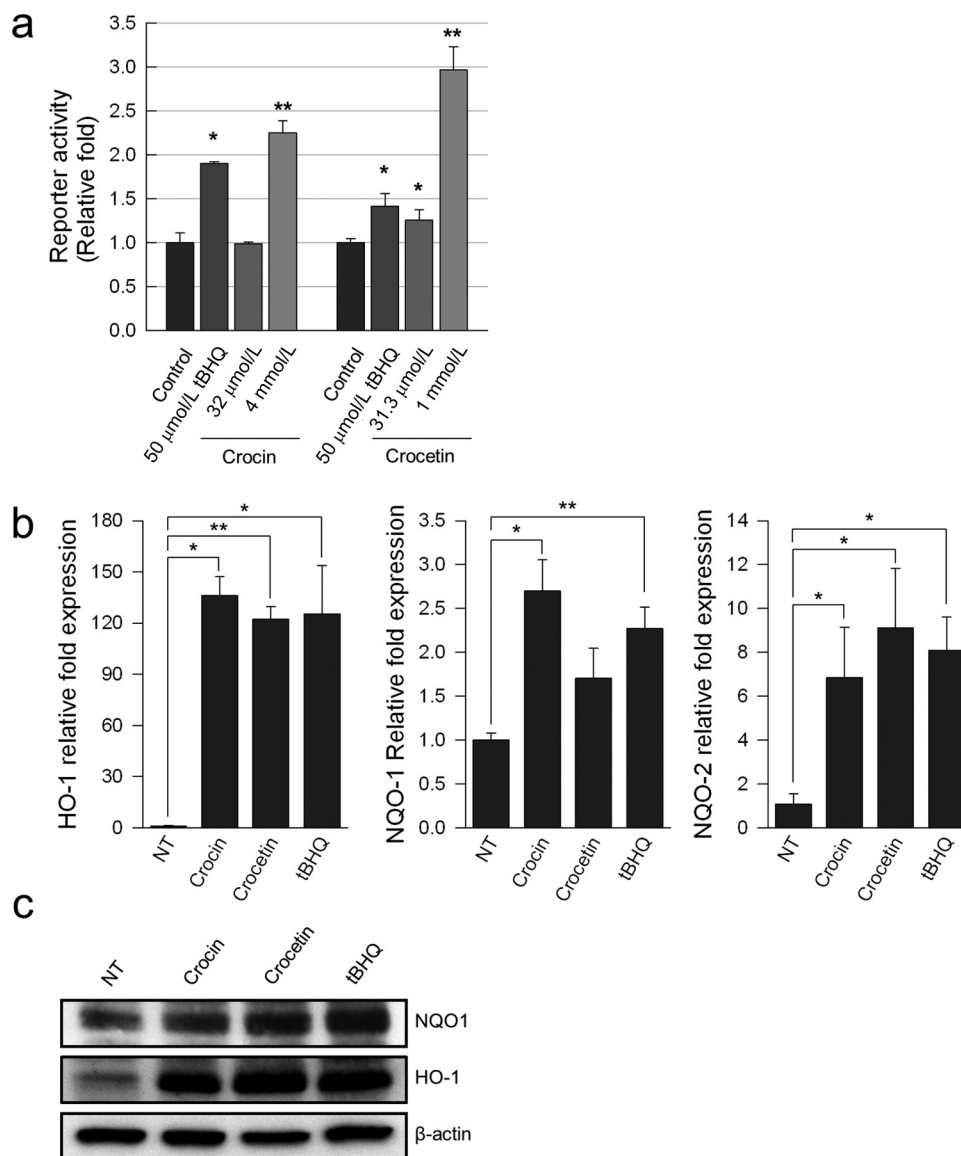
(5.0 mmol/L or 7.0 mmol/L) was added (Fig. 2*b*). This coincides with the lack of ROS noticed in HeLa cells treated with crocin (Fig. 2*a*). Therefore, the crocetin-mediated induction of ROS is a possible mechanism for the anticancer activity of crocetin, whereas crocin has a different mechanism than crocetin for reducing viability of HeLa cells.

### Crocin/crocetin-mediated Nrf2 activation

The cellular level of Nrf2 was monitored with a luciferase assay after 6 h of treatment of crocin or crocetin to investigate how the increased level of ROS induced the cytotoxicity observed in HeLa cells. Nrf2 is known to be activated by antioxidants such as *tert*-butylhydroquinone (tBHQ). Similar to tBHQ, both crocetin and crocin activated Nrf2 at higher concentration in HeLa cells (Fig. 3*a*). The extent to which Nrf2 was activated by crocin and crocetin was different: a 2.3-fold increase of luciferase activity was detected in HeLa cells treated with 4.0 mmol/L crocin, whereas a

3.0-fold increase of activity was observed in HeLa cells treated with 1.0 mmol/L crocetin. To determine the Nrf2-mediated expression of ARE, transcription levels of HO-1, NQO1, and NQO2 in HeLa cells treated with 4.0 mmol/L crocin or 1.0 mmol/L crocetin were quantified using real-time PCR. As expected, mRNA levels of HO-1, NQO1, and NQO2 increased in the HeLa cells treated with crocin or crocetin (Fig. 3*b*). Notably, the expression level of HO-1 increased over 120-fold, NQO1 increased 1.7- to 2.7-fold, and NQO2 increased 6.8- to 9.1-fold compared with the expression of nontreated cells. This result coincides with the result obtained by Western blot analysis performed for HO-1 and NQO1 (Fig. 3*c*).

Inhibitory activity of crocin and crocetin on the expression of LDHA in HeLa cells

Although the direct relationship between the activation of the ARE transcription system and the inhibitory effect on the viability of cancer cells is still unclear (Linnewiel et al. 2009), we assumed

Fig. 4. Inhibition of LDHA in HeLa cells treated by crocin or crocetin. Cells were treated with crocin or crocetin for 24 h following overnight incubation. (*a*) Protein expression levels of LDHA were analyzed by Western blot. Lamin-A was a control target gene to ensure equal loading. (*b*) LDHA activity was measured with whole-cell lysates using 1 mmol/L pyruvate and 0.5 mmol/L NADH as a substrate and a cofactor, respectively. Values are the means ± SD of 3 experiments, each performed in triplicate (*$p < 0.05$, **$p < 0.001$). NT: no treatment.
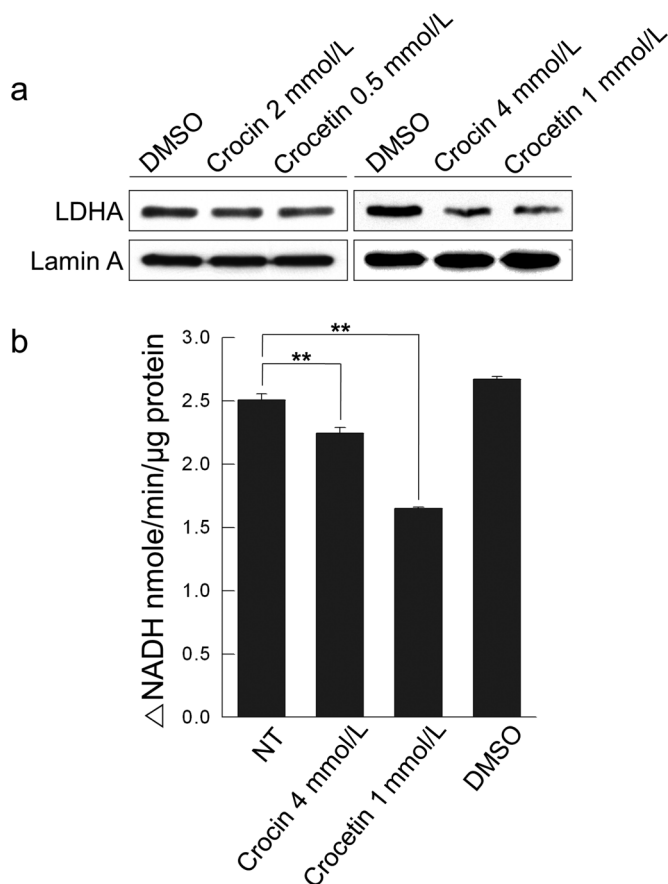
that the LDHA inhibitory activity of crocin and crocetin may account for the observed cytotoxicity of the HeLa cells overexpressing ARE and Nrf2. Therefore, the LDHA inhibitory activity of crocin and crocetin was determined by measuring both the protein expression and activity of the LDHA of HeLa cells treated with crocin (2.0 and 4.0 mmol/L) or crocetin (0.5 and 1.0 mmol/L) as well as 2 controls (no treatment and vehicle control DMSO). Immunoblotting of LDHA showed a dose-dependent decrease in the LDHA expression level of the HeLa cells treated with crocin or crocetin (Fig. 4*a*). However, in terms of efficacy, crocetin significantly decreased LDHA expression levels compared to crocin. This reduced expression of LDHA coincided with the measured reduced activity of LDHA in HeLa cells treated with 4 mmol/L crocin and 1 mmol/L crocetin (Fig. 4*b*). 1 mmol/L crocetin decreased the LDHA activity by 34.2% compared to the control (Fig. 4*b*), whereas 4.0 mmol/L crocin decreased the LDHA activity by 10.5% compared to the control.

Discussion

Saffron, a traditional coloration and medical reagent source, has received interest because of its beneficial biological activities, including its anticancer activity. Its anticancer effect stems from its constituent carotenoids, including crocin, picocrocin, safranal, and crocetin. Studies into the structure–activity relationship of

carotenoids have indicated that carotenoids with more complex structures, such as the ones with hydroxyl, aldehyde, or carboxyl groups on their chromophores, have a tendency to show higher antioxidant activities (Albrecht et al. 2000). In particular, the type and position of the polar functional groups, such as the carboxylic or aldehyde groups, were significant factors controlling cellular signal or metabolic pathways (Linnewiel et al. 2009; Ben-Dor et al. 2005). This is the case for the various cytotoxic activities exerted by crocetin and crocin. So far, it is not certain whether crocin or crocetin undergo further oxidative cleavage by carotenoid cleavage dioxygenases in cells, as in the case of other carotenoids (such as lycopene or β-carotene), which are cleaved into bioactive vitamins or signaling molecules (Wyss 2004). In addition, the membrane permeability of crocin and crocetin should be considered a reason for the observed cytotoxicity. To answer the effect of permeability of crocin and crocetin, a efficient delivery system, such as nanoliposomal vehicles to cancer cell lines (Mousavi et al. 2011), is needed.

Given the different cytotoxic activities of crocin and crocetin, the structure–activity relationship was investigated by measuring cellular ROS level, Nrf2 activation, and LDHA activity in HeLa cells. Although a previous study reported that saffron extracts did not induce ROS significantly in HeLa cells (Tavakkol-Afshari et al. 2008), crocetin induced a significant amount of ROS in HeLa cells, whereas crocin did not (Fig. 2*a*). The role of the induced ROS in HeLa cells treated with crocetin was further confirmed by applying a ROS-scavenger, NAC, to the HeLa cells treated with crocetin or crocin. As expected, NAC significantly increased the viability of the HeLa cells treated with crocetin by 40% by scavenging ROS, whereas the viability of the control HeLa cells did not change. The co-treatment of crocin and NAC also increased the viability of the HeLa cells by approximately 10%, implying that crocin also induced ROS by a small amount. Therefore, the combined results suggest that the highly induced ROS can account for the higher anticancer activities for crocetin versus crocin. Because the cellular ROS levels induced by crocin were not significant, the observed cytotoxic activity of crocin cannot be explained by the ROS induced by crocetin. Therefore, we postulate that crocin has other mechanisms for the observed cytotoxic activity rather than the observed cellular induction of ROS.

Nrf2 is the major transcription factor of the antioxidant response and phase II detoxifying system, and Nrf2 activation is regarded as a protective response against oxidative stress (Ohta et al. 2008; Wang et al. 2008). Like chemopreventive phytochemicals, such as curcumin and caffeic acid phenethyl ester (Surh 2003), both crocin and crocetin activated Nrf2 and upregulated AREs such as NQO1, NQO2, and HO-1. However, in a similar way to the induction pattern of ROS in the HeLa cells treated with crocetin and crocin, crocetin activated more Nrf2 than crocin in HeLa cells. Therefore, crocin or crocetin induced the cytotoxicity of cancer cell lines by influencing other cellular process related to activation of Nrf2 as previously reported for other bioactive carotenoids, which reduced the viability of cancer cell lines by the activation of Nrf2 (Linnewiel et al. 2009). However, Nrf2 activation could be caused by either crocetin/crocin or ROS generation, or possibly by both. Therefore, further studies are required to ascertain the relationship between Nrf2 activation and cancer cell death.

Finally, LDHA inhibition is one of the chemopreventive treatments that represses lactate formation, lowers membrane potential and the ATP levels of mitochondria, and consequently induces oxidative stresses (Fantin et al. 2006) in cancer cells undergoing hypoxia (Zhou et al. 2010). Further, cancer cells preferentially use aerobic glycolysis even in the presence of oxygen to fulfill cellular metabolic demands, namely the Warburg effect (Bayley and Devilee 2012). This unique metabolism is thought to be shielded from ROS produced in the mitochondria. Indeed, knockdown of LDHA, a mediator of aerobic glycolysis, was reported to elevate mitochon-

drial ROS production and a concomitant decrease in cell proliferation (Arseneault et al. 2013). The tendency of Nrf2 to be activated in crocin/crocetin-treated HeLa cells was consistent with LDHA inhibition, suggesting that the ROS induced by LDHA inhibition contributed Nrf2 activation in crocin/crocetin-treated HeLa cells.

In conclusion, crocetin and crocin, both major constituent carotenoids in saffron, have cytotoxic activities at different IC_{50} values, although their respective mechanisms for inducing cancer cell death seem to be different. We presume that crocetin induces more cellular ROS than crocin does primarily by repressing the expression and (or) activity of LDHA in cancer cell lines for hypoxia. Unlike the clear induction of cellular oxidative stress by crocetin, cellular oxidative stress by crocin is relatively mild and weak. Therefore, we assume that the cytotoxic activity of crocin is based on other cellular mechanisms, including its antiproliferation and apoptogenic properties as reported in other studies (Mousavi et al. 2011; Sun et al. 2011; Samarghandian et al. 2010). Elucidation of different cytotoxic mechanisms for crocin and crocetin would help us to understand the relationship of anticancer effects and structural diversity, and eventually make the targeted structure of carotenoid production or treatment available. In particular, inhibitory activity of crocin/crocetin on LDHA in cancer cells could serve as a basis for a study of anticancer activities of carotenoids. In addition, this encourages the need for investigating the biological activities of precursors of natural biologically active compounds, including terpenoids or flavonoids.

Acknowledgements

References

Albrecht, M., Takaichi, S., Steiger, S., Wang, Z.Y., and Sandmann, G. 2000. Novel hydroxycarotenoids with improved antioxidative properties produced by gene combination in *Escherichia coli*. Nat. Biotechnol. **18**(8): 843–846. doi:10.1038/78443. PMID:10932152.

Arseneault, R., Chien, A., Newington, J.T., Rappon, T., Harris, R., and Cumming, R.C. 2013. Attenuation of LDHA expression in cancer cells leads to redox-dependent alterations in cytoskeletal structure and cell migration. Cancer Lett. **338**(2): 255–266. doi:10.1016/j.canlet.2013.03.034. PMID:23583676.

Aung, H.H, Wang, C.Z., Ni, M., Fishbein, A., Mehendale, S.R., Xie, J.T., et al. 2007. Crocin from *Crocus sativus* possesses significant anti-proliferation effects on human colorectal cancer cells. Exp. Oncol. **29**(3): 175–180. PMID:18004240.

Bayley, J.P., and Devilee, P. 2012. The Warburg effect in 2012. Curr. Opin. Oncol. **24**(1): 62–67. doi:10.1097/CCO.0b013e32834deb9e. PMID:22123234.

Ben-Dor, A., Steiner, M., Gheber, L., Danilenko, M., Dubi, N., Linnewiel, K., et al. 2005. Carotenoids activate the antioxidant response element transcription system. Mol. Cancer Ther. **4**(1): 177–186. PMID:15657364.

Escribano, J., Alonso, G.L., Coca-Prados, M., and Fernández, J.A. 1996. Crocin, safranal and picrocrocin from saffron (*Crocus sativus* L.) inhibit the growth of human cancer cells *in vitro*. Cancer Lett. **100**(1–2): 23–30. doi:10.1016/0304-3835(95)04067-6. PMID:8620447.

Fantin, V.R., St-Pierre, J., and Leder, P. 2006. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell, **9**(6): 425–434. doi:10.1016/j.ccr.2006.04.023. PMID:16766262.

Gutheil, W.G., Reed, G., Ray, A., Anant, S., and Dhar, A. 2012. Crocetin: An agent derived from saffron for prevention and therapy for cancer. Curr. Pharm. Biotechnol. **13**(1): 173–179. doi:10.2174/138920112798868566. PMID:21466430.

Holt, N.E., Zigmantas, D., Valkunas, L., Li, X.P., Niyogi, K.K., and Fleming, G.R. 2005. Carotenoid cation formation and the regulation of photosynthetic light harvesting. Science, **307**(5708): 433–436. doi:10.1126/science.1105833. PMID:15662017.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., et al. 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun. **236**(2): 313–322. doi:10.1006/bbrc.1997.6943. PMID:9240432.

Kanakis, C.D., Tarantilis, P.A., Tajmir-Riahi, H.A., and Polissiou, M.G. 2007*a*. Interaction of tRNA with safranal, crocetin, and dimethylcrocetin. J. Biomol. Struct. Dyn. **24**(6): 537–545. doi:10.1080/07391102.2007.10507142. PMID:17508775.

Kanakis, C.D., Tarantilis, P.A., Tajmir-Riahi, H.A., and Polissiou, M.G. 2007*b*. DNA interaction with saffron's secondary metabolites safranal, crocetin, and dimethylcrocetin. DNA Cell Biol. **26**(1): 63–70. doi:10.1089/dna.2006.0529. PMID:17263598.

Lee, P.C., Momen, A.Z.R., Mijts, B.N., and Schmidt-Dannert, C. 2003. Biosynthesis of structurally novel carotenoids in *Escherichia coli*. Chem. Biol. **10**(5): 453–462. doi:10.1016/S1074-5521(03)00103-0. PMID:12770827.

Linnewiel, K., Ernst, H., Caris-Veyrat, C., Ben-Dor, A., Kampf, A., Salman, H., et al. 2009. Structure activity relationship of carotenoid derivatives in activation of the electrophile/antioxidant response element transcription system. Free Radic. Biol. Med. **47**(5): 659–667. doi:10.1016/j.freeradbiomed.2009.06.008. PMID:19524036.

Mousavi, S.H., Moallem, S.A., Mehri, S., Shahsavand, S., Nassirli, H., and Malaekeh-Nikouei, B. 2011. Improvement of cytotoxic and apoptogenic properties of crocin in cancer cell lines by its nanoliposomal form. Pharm. Biol. **49**(10): 1039–1045. doi:10.3109/13880209.2011.563315. PMID:21936628.

Ohta, T., Iijima, K., Miyamoto, M., Nakahara, I., Tanaka, H., Ohtsuji, M., et al. 2008. Loss of keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. Cancer Res. **68**(5): 1303–1309. doi:10.1158/0008-5472.CAN-07-5003. PMID:18316592.

Samarghandian, S., Boskabady, M.H., and Davoodi, S. 2010. Use of *in vitro* assays to assess the potential antiproliferative and cytotoxic effects of saffron (*Crocus sativus* L.) in human lung cancer cell line. Pharmacogn. Mag. **6**(24): 309–314. doi:10.4103/0973-1296.71799. PMID:21120034.

Sharoni, Y., Linnewiel-Hermoni, K., Khanin, M., Salman, H., Veprik, A., Danilenko, M., and Levy, J. 2012. Carotenoids and apocarotenoids in cellular signaling related to cancer: A review. Mol. Nutr. Food Res. **56**(2): 259–269. doi:10.1002/mnfr.201100311. PMID:22102431.

Sun, J., Xu, X.M., Ni, C.Z., Zhang, H., Li, X.Y., Zhang, C.L., et al. 2011. Crocin inhibits proliferation and nucleic acid synthesis and induces apoptosis in the human tongue squamous cell carcinoma cell line tca8113. Asian Pac. J. Cancer Prev. **12**(10): 2679–2683. PMID:22320974.

Surh, Y.J. 2003. Cancer chemoprevention with dietary phytochemicals. Nat. Rev. Cancer, **3**(10): 768–780. doi:10.1038/nrc1189. PMID:14570043.

Tavakkol-Afshari, J., Brook, A., and Mousavi, S.H. 2008. Study of cytotoxic and apoptogenic properties of saffron extract in human cancer cell lines. Food Chem. Toxicol. **46**(11): 3443–3447. doi:10.1016/j.fct.2008.08.018. PMID:18790714.

Wang, X.J., Sun, Z., Villeneuve, N.F., Zhang, S., Zhao, F., Li, Y., et al. 2008. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. Carcinogenesis, **29**(6): 1235–1243. doi:10.1093/carcin/bgn095. PMID:18413364.

Wyss, A. 2004. Carotene oxygenases: A new family of double bond cleavage enzymes. J. Nutr. **134**(1): 246S-250S. PMID:14704328.

Zhou, M., Zhao, Y., Ding, Y., Liu, H., Liu, Z., Fodstad, O., et al. 2010. Warburg effect in chemosensitivity: Targeting lactate dehydrogenase-A re-sensitizes Taxol-resistant cancer cells to taxol. Mol. Cancer, **9**(1): 33. doi:10.1186/1476-4598-9-33. PMID:20144215.