

RESEARCH COMMUNICATION

Antiproliferative Effects of Crocin in HepG2 Cells by Telomerase Inhibition and hTERT Down-Regulation

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Abstract

Crocin, the main pigment of *Crocus sativus* L., has been shown to have antiproliferative effects on cancer cells, but the involved mechanisms are only poorly understood. This study focused on the probable effect of crocin on the immortality of hepatic cancer cells. Cytotoxicity of crocin (IC₅₀ 3 mg/ml) in hepatocarcinoma HepG2 cells was determined after 48 h by neutral red uptake assay and MTT test. Immortality was investigated through quantification of relative telomerase activity with a quantitative real-time PCR-based telomerase repeat amplification protocol (qTRAP). Telomerase activity in 0.5 µg protein extract of HepG2 cells treated with 3 mg/ml crocin was reduced to about 51% as compared to untreated control cells. Two mechanisms of inhibition, i.e. interaction of crocin with telomeric quadruplex sequences and down regulation of hTERT expression, were examined using FRET analysis to measure melting temperature of a synthetic telomeric oligonucleotide in the presence of crocin and quantitative real-time RT-PCR, respectively. No significant changes were observed in the T_m telomeric oligonucleotides, while the relative expression level of the catalytic subunit of telomerase (hTERT) gene showed a 60% decrease as compared to untreated control cells. In conclusion, telomerase activity of HepG2 cells decreases after treatment with crocin, which is probably caused by down-regulation of the expression of the catalytic subunit of the enzyme.

Keywords: Crocin - telomerase - hTERT - hepatocarcinoma

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Introduction

Cancer is one of the most important health problems around the world, not only in older patients but also in children. Chemoprevention of cancer especially by natural compounds is a promising strategy against cancer initiation. In this regard natural compounds with strong antioxidative, hepatoprotective and anti-inflammatory effects are interesting candidates to evaluate their ability of influence the initiation and growth of tumors.

Food additives in particular might be valuable candidates for a deeper investigation. Saffron, obtained from the dried stigma of *Crocus sativus* L., is an important spice rich in terpenoids. It is commonly consumed in different parts of the world and used as a medicinal drug to treat several health disorders. Promising and selective anti-cancer effects of saffron have been observed *in vitro* and *in vivo* (Schmidt et al., 2007). Saffron extracts were shown to inhibit papilloma growth, decline the incidence of squamous cell carcinoma and soft tissue sarcoma in mice. Furthermore, the extracts inhibit the synthesis of nucleic acids but not of proteins (Nair et al., 1995). Based on the Ames test saffron was shown to be nontoxic, non-mutagenic, non-antimutagenic and non-comutagenic (Abdullaev et al., 2002). Crocin appears to exhibit an anticarcinogenic activity (Konoshima & Takasaki, 2003).

This compound has recently been shown to reduce haematological cytotoxicity of diazinon in rats, while does not prevent its genotoxicity (Hariri et al., 2011). However, saffron is only safe in small quantities typically used in spices and higher amounts can be dangerous and during pregnancy can induce an abortion. In addition to non-toxic carotenoids, saffron contains picrocrocin which is hydrolysed to give safranal that can bind to amino groups of proteins and DNA bases (Wink & Van, 2008). Studies using animal models and cultured human malignant cell lines have demonstrated anti-tumor and cancer preventive activities of saffron and its main ingredients (Abdullaev et al., 2004).

Among the earlier researches on crocin, crocetin, safranal and picrocrocin extracted from saffron, it is suggested that crocetin does not show cytotoxic effects while cells treated with crocin exhibit apoptosis induction and because of water-solubility and high growth inhibitory effect, crocin is the most promising saffron constituent as a cancer therapeutic agent (Escribano et al., 1996). A neuroprotective effect of crocin on acrylamide-induced cytotoxicity in PC12 cells has been reported recently which was explained with its antioxidant properties again (Mehri et al., 2012). Another possible mechanism of crocin in its neuroprotective properties could be inhibition of protein aggregation and fibrillar formation (Ebrahim-

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Habibi et al., 2010).

In this project we have focused on growth limiting effects of crocin, the major constituent and the main dark red pigment of the saffron in hepatocarcinoma cells (HepG2). Crocin is a diester formed from the disaccharide gentiobiose and the dicarboxylic acid crocetin. An antidepressant property has been attributed to crocin (Akhondzadeh et al., 2004). It is well known as a potent antioxidant (Ochiai et al., 2006; Papandreou et al., 2006; Zheng et al., 2006), so that it received so much attention against oxidative stress related disorders. In chronically stressed mouse model it prevents impairment of learning and memory as well as lipid peroxidation products. Crocin protects oxidative damage to the hippocampus and reduces plasma level of corticosterone after the end of stress (Ghadroost et al., 2011). Again in mouse model crocin improve spatial cognitive abilities following chronic cerebral hypoperfusion that may be related to its antioxidant effects (Hosseinzadeh et al., 2012).

Cancer cells in spite of normal somatic cells are immortal, and it is mainly because of the activity of a special RNA-dependent DNA polymerase, called telomerase. This enzyme serves to maintain the telomeric repeats at eukaryotic chromosomes' ends (Blackburn & Greider, 1995), by compensating telomere attrition which is normally occur in proliferating cells due to incomplete DNA replication by DNA polymerases. Telomerase is down-regulated in many adult somatic cells but is drastically over expressed in almost 90% of different kind of human cancers by up to a hundred-fold (Hiyama et al., 1995), conferring a strong selective advantage for continued growth to malignant cells (Smith et al., 2003). Therefore, telomerase has been considered as a key enzyme in cell immortality (Huffman et al., 2000) that also plays an important role in the telomere-capping function (Blackburn, 2001). It has been proposed that a telomerase inhibitor could complement classical cancer chemotherapeutics (Shay & Wright, 2002). Critically short telomeres induce proliferative senescence, apoptosis or continued proliferation accompanied by genomic instability (Ludwig et al., 2001). Many studies indicate that inhibition of telomerase can affect the survival of cancer cells without long-term treatment of the cells because of apoptosis induction (Hahn et al., 1999; Yi-hsin & Jing-er, 2005). The inhibition of telomerase by various strategies such as antisense oligonucleotides (Kondo et al., 1998), small-molecule inhibitors (Damm et al., 2001) ribozymes (Nosrati et al., 2004) and RNA interference (Li et al., 2004) has been proposed.

In the present study, crocin was applied to hepatocarcinoma HepG2 cells. The observed cytotoxicity could be explained by a probable inhibition of telomerase. A down-regulation of hTERT could be established while quadruplex formation of crocin with telomeres is less likely.

Materials and Methods

Cell culture

The HepG2 cell line (ACC 180 from DSMZ) was maintained in 75 cm² culture flasks in RPMI-1640

culture medium (Gibco, Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (Biochrome, Berlin, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in 5% CO₂ at 37 °C and 100% humidity. Media were changed every 48 h, and the cells were subcultured after 5 - 6 days using trypsin-EDTA. To measure the cytotoxicity of crocin (crocetin digentiobiose ester, 17304, grade Standard Fluka; dissolved in distilled water), neutral red uptake test and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) purchased from Sigma-Aldrich were employed.

Cytotoxicity test

Exponentially growing HepG2 cells were seeded in 96 well plates with 10,000 cells per well. Medium was changed after 24 h with the fresh medium plus various concentrations of crocin. In neutral red uptake assay after 48 h treatment, the cells were washed with PBS and incubated in fresh medium including 50 µg/ml neutral red (Borenfreund et al., 1990). The medium was removed after three h incubation and the dye dissolved in 200 µl of isopropyl alcohol containing 0.04 M HCl. The absorbance was measured at 570 nm. The test was repeated four times each in triplicate and the 50% lethal dosage, LD₅₀, values were calculated from dose-response curves. For MTT test, 0.5 mg/ml MTT was added to each well of 48 h treated cells with various concentrations of aqueous solutions of crocin (Mosman, 1983). After 4 hours the reduced purple formazan product of MTT was dissolved by adding 200 µl of solvent containing 10% SDS and 1% acetic acid in dimethylsulfoxide. Absorbance measurements and LD₅₀ calculations were carried out as in the neutral red uptake test.

Assay of telomerase activity

HepG2 cells after 48 hours incubation with various concentrations of crocin were trypsinized and washed with cold PBS, and then incubated in lysis buffer as previously described (Kazemi Noureini et al., 2004). The lysates were centrifuged at 16,000 g for 30 min and protein concentrations of the supernatant were measured using the Bradford assay (Bradford, 1976). Telomerase activity was determined using 0.5 µg protein of each sample, isolated from the cells, in duplicates. A negative control was included which consisted of untreated control extracts inactivated by boiling and/or RNase A treatment. For a quantitative measurement a real-time protocol was used according to Hou et al. (2001), and a conventional TRAP test also was applied as reported before (Kazemi Noureini et al., 2004) to visualize the telomerase products. The activity of untreated control cells was considered as 100%.

RNA isolation, reverse transcription and real-time RT-PCR

Total RNA from HepG2 cells that had been treated with various concentrations of crocin below the LD₅₀, was isolated using RNeasy Mini Kit (Qiagen; Germany) according to the manufacturer's instruction and treated with DNase I (RNase-Free DNase Set, Qiagen; Germany). First strand cDNA synthesis was performed according to the protocol suggested for the Reverse Transcription System

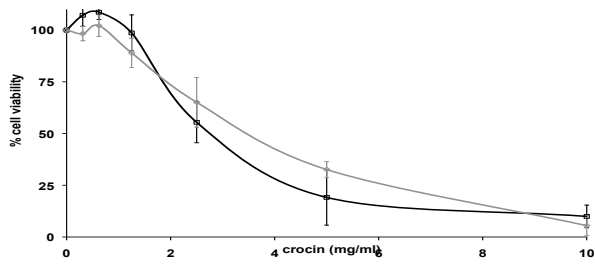


Figure 1. Viability Test using Neutral Red Uptake (Black) and MTT (Gray) to Measure IC_{50} Concentration of Crocin in HepG2 Cells After 48 h. The mean \pm SD values of four repetitions including triplicates for each point are presented

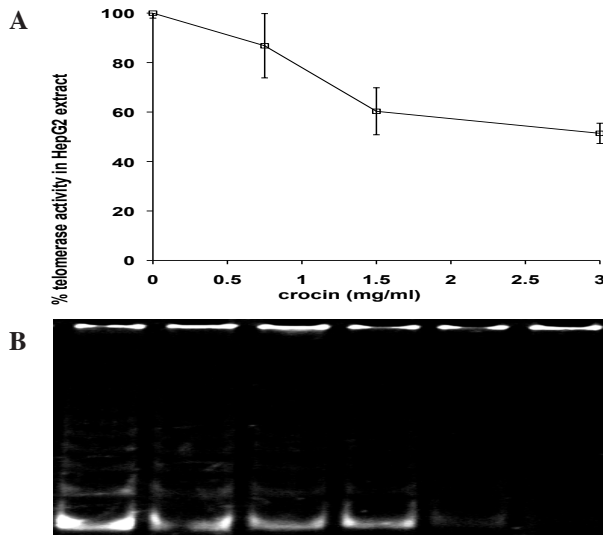


Figure 2. A) Telomerase Activity Measurements of HepG2 Extracts after 48h Treatment with Different concentration of crocin using qTRAP. Activity of untreated cells was regarded as 100% (mean \pm SD). **B) TRAP products of crocin-treated HepG2 extracts resolved on a 15% native acrylamide gel.** From left to right: 0, 0.05, 0.25, 1.5, 3 mg/ml crocin and negative control

(Promega Corporation; Madison, USA). Quantitative real-time RT-PCR was carried out in 20 μ l reaction volume containing 0.2 μ g of cDNA, 1x HotStar Master SYBR Green I, 0.5 μ M of each primer as previously described (Kazemi & Wink, 2009). Relative expression of hTERT as compared to the housekeeping gene β 2-microglobulin as was determined from three repetitions including two measurements for each of the duplicated samples for the defined concentrations of crocin.

FRET analysis of synthetic dual labelled synthetic telomeric oligonucleotide

To estimate the potential of crocin to directly bind to telomeres, we evaluated melting temperature of a synthetic dual labeled oligonucleotide F21T 5' fluorescein-GGG(TTAGGG)3-3' TAMRA 0.2 (Eurofins MWG Operon, Germany) in 0.2 μ M final concentration in a 25 μ L of 0.1 M potassium chloride, 10 mM sodium cacodylate (pH 7.2) buffer according to (Mergny et al., 2001) using a real-time PCR machine Rotor Gene 3000 (Corbet-Qiagen). Briefly, the oligonucleotide was heated 10 min at 90 $^{\circ}$ C and cooled on ice and different amounts of crocin were added to achieve the desired concentration.

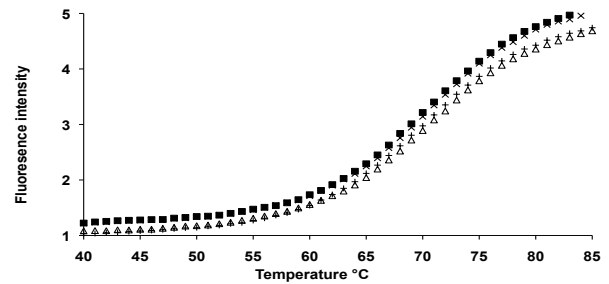


Figure 3. Influence of Crocin on Melting Temperature of Telomeric DNA. Thermal FRET analysis results based on fluorescence intensity of F21T telomeric oligonucleotide after 2 h incubation with (■, 0), (×, 0.75), (+, 1.5) and (Δ, 3) mg/ml crocin

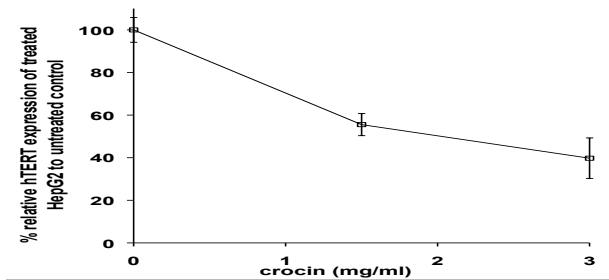


Figure 4. Transcription Level of hTERT using Real-Time PCR Experiments. Activity of the house keeping gene beta-microglobulin was set to 100%. The mean \pm SD values of three logical repeats of each duplicated points are presented

After 2 h incubation at 37 $^{\circ}$ C, a heating program starting from 37 $^{\circ}$ C to 95 $^{\circ}$ C by a rate of 0.5 $^{\circ}$ C per minute was run and T1/2 was compared among different concentrations of crocin (Stewart, 1968).

Statistical analysis

Statistical analysis was performed by using standard student (t) test and a P < 0.05 was considered as the cut off for significant difference.

Results

Cytotoxicity determinations are based neural red uptake and MTT tests. Crocin shows only low cytotoxicity with IC_{50} values of 2.75 mg/ml (neural red assay) and 3.25 mg/ml (MTT assay) after 48 hours (Figure 1). A small increase in viable cell numbers was seen in very low concentrations of crocin (0.3 and 0.6 mg/ml), which possibly is explained with antioxidant properties of polyunsaturated terpenoids such as crocin and its metabolites (Pham et al., 2000).

Quantitative real-time telomerase repeat amplification protocol (qTRAP) measurements in equal amount of protein extracts (0.5 μ g) showed a considerable reduction of telomerase activity to around 51% of untreated control cells after 48 h treatment with 3 mg/ml crocin. Telomerase activity reduction occurs in a concentration dependent manner (Figure 2).

A potential interaction of crocin with telomeric sequences was tested by measuring melting points of a synthetic telomeric oligonucleotide using FRET. Almost no increase in T1/2 was detected even in presence of 3 mg/ml crocin (Figure 3).

It has been shown before that telomerase activity is mainly regulated through the expression level of its catalytic subunit. Levels of mRNA of hTERT were determined with RT-PCR in control and treated samples. As compared to the expression of beta-microglobulin, mRNA levels of hTERT were reduced 60% after 48 h as compared to untreated controls (Figure 4).

The reduction of enzyme activity and gene transcription show a similar but not identical dose dependence, a probable cause for this difference could be the short half-life of the hTERT mRNA (Gunes et al., 2000), as compared to the more stable telomerase ribonucleoprotein.

Discussion

The decrease of telomerase activity in HepG2 cell treated with crocin agrees with a recent publication showing that crocin exhibits anti-proliferative effects in human colorectal cancer cell lines while not affecting normal cells (Aung et al., 2007). In addition, crocin and crocetin were shown to inhibit breast cancer cell proliferation (Chryssanthi et al., 2007). Furthermore in vitro and in vivo experiments have already confirmed a concentration- and time-dependent growth inhibition by crocin in xenograft mouse models (Bakshi et al., 2009). A clinically useful effect of crocin in stimulation of dendritic cells that can particularly enhance the proliferation of T cells in mononuclear cells isolated from bone marrow of leukemia children

Among many different probable mechanisms of anti-proliferative effect of saffron extract, a significant inhibition in the synthesis of nucleic acids but not of proteins (by dimethyl-crocetin) was related to retardation of papilloma growth and disruption of DNA-protein interactions, e.g. topoisomerases II (Nair et al., 1995). This property however could not interfere with our telomerase activity determinations, because in TRAP assay experiments which relies on PCR the compound is washed out with PBS at least twice before the actual assay and it get diluted at least 10 times more in reaction buffer.

It should be recalled that a saffron extract contains picrotoxin and safranal, an aldehyde which can directly alkylate DNA. Such a pharmacophoric group is absent in crocin. Reducing telomerase activity also agrees with another report indicating that crocin derivatives were more active in tumor cell colony formation in an in vitro assay study than other carotenoids (Bakshi et al., 2010; Sun et al., 2011).

In conclusion, to our knowledge this is the first report showing an inhibitory effect of crocin on telomerase activity that is probably by down regulating transcription of hTERT gene. As telomerase activity is significantly higher in cancer cells and almost undetectable in normal cells, it might be a selective target in cancer therapy. Its inhibition would selectively suppress tumor growth without considerable effects on normal cells. Another study has shown improvement of cytotoxic and apoptogenic properties of crocin in cancer cell lines, HeLa and MCF-7, using its nanoliposomal form, which make it more efficient with considerably lower IC₅₀ values

(Mousavi et al., 2011). Therefore crocin is a promising candidate in cancer chemoprevention which might be improved by additional drug design.

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