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Crocin induced apoptosis through p53-dependent pathway in C6 glioma Cells

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ABSTRACT

Crocin has been shown to induce apoptosis in C6 glioma cells. However, the signaling pathway of its apoptotic effects remains unknown. In this study, the apoptotic effect of crocin on C6 glioma cells was investigated. Results demonstrated that crocin exhibited strong suppressing effect on C6 cells. Furthermore, crocin induced apoptosis in C6 cells was clearly verified by the appearance of apoptotic cells. Moreover, significant increase in the levels of apoptosis-related p53 signal was observed in crocin treated cells. Taken together, crocin displays effective inhibitory effects on the growth of C6 cells and stimulates the expression of p53. Our results suggest that crocin-induced apoptosis in C6 cells is mediated through the activation of p53. It implies that crocin could be a useful chemotherapeutical agent for treatment of glioma.

Keywords: Glioma, Drug Therapy, Crocin, Apoptosis, p53.

I. INTRODUCTION

Crocin is digentiobiosyl ester of crocetin–α-crocin, a carotenoid chemical compound that is found in the flowers crocus. Crocin is a major component of saffron spice, the chemical ingredient primarily responsible for the color of saffron. Chemically, crocin is the diester formed from the disaccharide gentiobiose and the dicarboxylic acid crocetin. Crocin is the major active ingredient of saffron underlying saffron's aroma and comprises more than 10% of dry saffron's mass. Crocin has been shown to have antioxidant, neuroprotective, anti-inflammatory, antidepressant and antiproliferative activities [1-6]. Crocin inhibits tumor growth and proliferation in different forms of cancers including colorectal, pancreatic, breast, and different leukemias indicating its potential as an anti-cancer chemotherapeutic[1-12] Crocin has been found to inhibit cell proliferation and invasion in C6 glioma cells, induce cell death and inhibits cellular clonogeneceity (Data submitted for publication). However the mechanism of the apoptotic effects of crocin in glioma inhibition remains unknown.

Apoptotic process is highly regulated and complex cellular program. Cellular state such as increased oxidative stress and DNA damage is capable of activating apoptosis; inactivation of this program constitutes a critical step in the process of gliomagenesis [13-14]. TP53 encodes a transcription factor p53 that regulates genes critical for cell cycle progression and apoptosis. During normal cellular conditions, p53 is transitory and short-

lived. In response to cellular stress p53 undergoes post-translational modifications and protein-protein interactions that enhance its stability and transcriptional activity. Stable form of p53 regulates transcription of various proapoptotic genes. p53 interacts with a number of anti-apoptotic proteins and inhibits their activity. Mutations that inactivate this axis are found in greater than 70% of gliomas cells. p53 is one of the major tumor suppressor proteins in gliomas [15]. Since crocin has anti proliferative effects, to ascertain whether p53 is involved in drug induced apoptosis present study was carried out to investigate the effect of crocin on expression of p53 in C6 glioma cells we addressed the question whether a natural therapy with crocin could induce apoptosis in C6 cells or not and to explore the effect of crocin treatment on p53 pathway in glioma.

II. MATERIALS AND METHODS

2.1 Reagents

PI (propidium iodide) was purchased from Sigma-Aldrich, protein quantification assay kit from Bio-Rad Laboratories, PVDF (polyvinylidene difluoride) membrane was brought from Millipore-USA. All other chemicals were of ultra pure grade and obtained from Sigma-Aldrich, or otherwise indicated. 100% HPLC purified crocin generously provided by Dr. Syed Sana *et al*, Centre of Research For Development, University of Kashmir.

2.2 Cell Culture

The studies were performed on C6 cell line; Rattus Norvegicus glioma-derived cell line. The cells were cultured, subcultured and cryopreserved as per instructions (ATCC/ CCL-107) in DMEM supplemented with 10% FBS and 100 units penicillin-streptomycin and maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere. IC -50 dose of crocin in C6 glioma cells was 15mg/ml as was evaluated through a series of assays (unpulished data).

2.3 Quantification of apoptosis by propidium iodide staining

After treatment, the culture medium was aspirated from each well and cells were washed gently twice with pre-chilled PBS (Phosphate-buffered saline) for 5 min each time followed by incubation with 5 µg/ml PI solution for 30 min in the dark. The cells were then washed with Tris buffer (50 mM Tris-HCl, pH7.3). Wells containing cells and treated with 0.2% Triton X-100 (10-15 min treatment) were taken to determine the maximum fluorescence signal from dead cells. Stained cells were monitored by the Floid cell imaging station (Life Technologies, California, USA). Six randomly chosen fields of view were observed after exposure to the condition. PI fluorescence intensities were measured using Image J software and presented as fold increase versus non-treated control.

2.4 Protein extraction and quantitation

After the required experimentation, culture media was removed from cell culture dishes and washed with pre-chilled PBS twice and scraped into lysis buffer (20 mM TrisCl (pH 8), 137 mM NaCl, 10% Glycerol, 1% Nonidet P-40, and 2 mM EDTA, 1mM PMSF, 5 mM NaF and phosphatase inhibitors). Cells were incubated at 4°C for 60 min. After the treatment samples were centrifuged at 12000 rpm for 10 min. The supernatants were transferred to freshly chilled Eppendorf tubes and protein samples were frozen at -80°C until further use. A Bio-Rad Laboratories protein assay kit based on Bradford's method was used for measuring protein concentration spectrophotometrically at 595nm as per instructions in the manual.

2.5 Western blotting analysis

Protein extract was preheated at 100°C for 5 min in reducing SDS sample buffer (50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue and 100 mM β-mercaptoethanol). Samples were adjusted to equal protein concentration and volume and subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) on 10-12% SDS-acrylamide gel with a discontinuous buffer system. Separated proteins were transferred to PVDF membrane by a semi-dry transfer method in accordance with the manufacturer's instructions (Hoefer-USA) for 1 hour at 200 mA. For immune-detection, the PVDF membrane was processed and blocked with 5% bovine serum albumin in PBS for three hours at room temperature. After blocking membranes were washed twice with PBST (0.2% tween-20) and then incubated with the respective specific primary antibodies; p53 (1:1000; Santa Cruz Biotechnologies Dallas Taxas, US). Anti β-actin (1:7000; Sigma-Aldrich) was used as loading control. After overnight incubation at 4° C temperature, membranes were washed thrice for 10 minutes with PBST (0.5 % tween). The secondary detection was performed using anti-mouse IgG Dylight 680 conjugate (1:10,000) secondary antibody. After washing three times for 10 minutes with PBST, Odyssey infrared detection system (LI-COR, Lincoln, NE, U.S.A.) was used to detect Western blots and band intensity. The results were represented as expression levels of specific gene normalized to β-actin relative to the normalized expression of the gene in control cells.

2.6 Statistical analysis

All the assays were repeated independently three times and values were expressed as Mean ± SD of three independent experiments. All data were analyzed by using One Way ANOVA by Dunnett's multiple comparisons tests using GraphPad Prism 5.

III. RESULTS

3.1 Crocin treatment induces cell damage accompanied by apoptosis

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We carried out PI staining to delineating morphological changes induced by stress from changes in morphology due to apoptosis. Results showed that the treatment of C6 cells with crocin significantly induced cell apoptosis in comparison to control group (fig 1A. In the control group, nuclei stained with PI were only ~15%, while as ~50% of the crocin treated cells were PI stained (fig 1B).

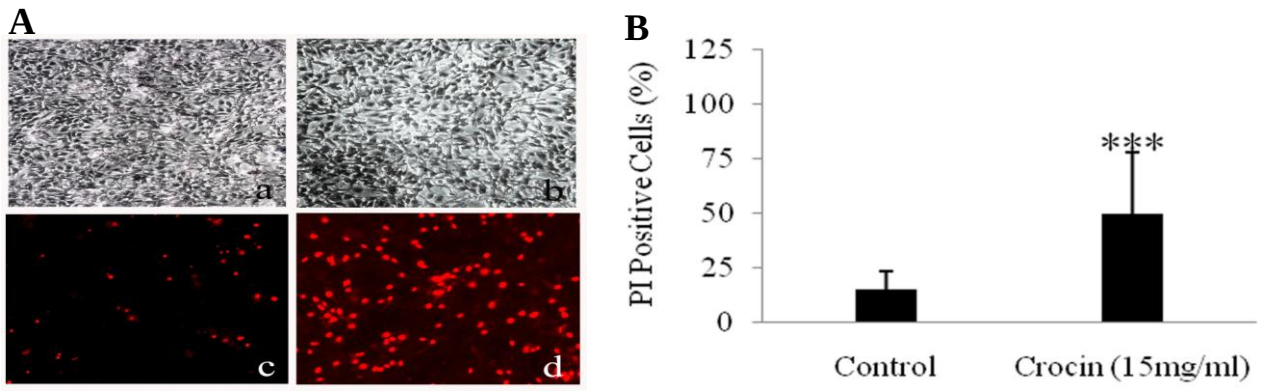

Figure 1: (A) Representative photomicrographs showing, BW images of (a) control, (b) crocin treated cells and respective fluorescent images stained with PI(c, d) (scale bar:100µm). (B) Fluorescence intensities measured using image J software, ~15% in control group, ~50% in crocin treated. (A) Representative photomicrographs showing, BW images of (a) control, (b) crocin treated cells and respective fluorescent images stained with PI(c, d) (scale bar:100µm). (B) Fluorescence intensities measured using image J software, ~15% in control group, ~50% in crocin treated cells.

3.2 Crocin induces apoptosis via up-regulation of p53 expression in C6 glioma cells

p53 is one of the major tumor suppressor proteins in gliomas. Glioma tumors retain wild type p53; hence ability to maintain the tumor phenotype depends on down regulated expression of p53. Since crocin induces apoptosis, to ascertain whether p53 is involved in drug induced apoptosis, Western blotting was employed. Our results showed that crocin treatment induced p53 expression in C6 glioma cells compared with the control (fig 2A). Furthermore, quantification of the protein blots after normalization with β-actin expression indicated that p53 expression was ~6 unit higher in samples treated with crocin , impeding growth and proliferation of C6 glioma cells (fig 2B).

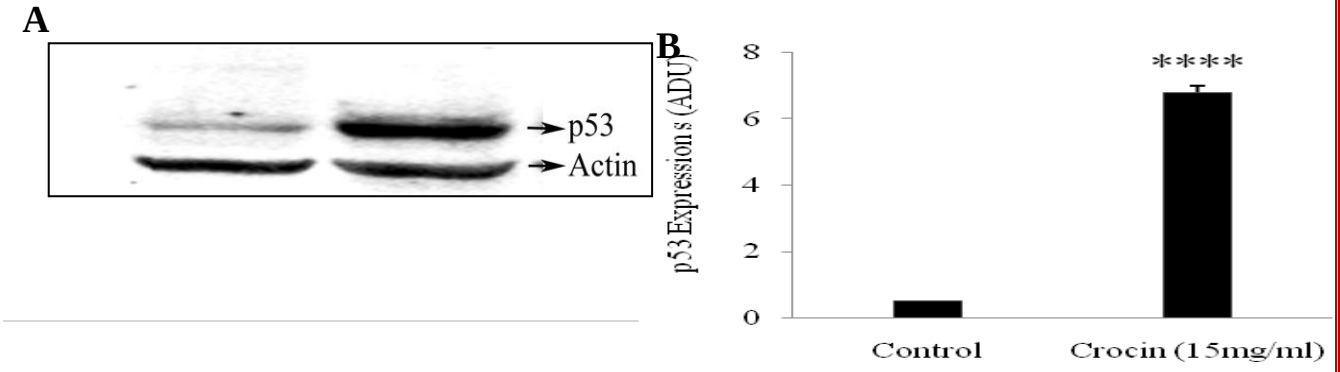

Figure 2: (A) Immuno blot showing the expression of p53 protein in C6 glioma cells: After treatment with crocin p53 protein is upregulated in co-treated group when compared with control, β-actin was used as loading control and expression in each group was normalized to β-actin relative to the normalized expression in control cells (B) Representative bar graphs depicting expression levels of p53 protein as shown by densitometric analysis in arbitrary densitometric units (ADU) in crocin treated C6 cells

IV. DISCUSSION

Crocin has been used in folk medicine since ages as anti-oxidative agent with a certain anticancer activity. It is known to have anti-mitogenic, anticarcinogenic activities in various malignancies [1-12]. Our previous studies showed that crocin treatment inhibited cell proliferation and induced cell death in C6 cells. Our results showed that crocin initiates the process of apoptosis in C6 glioma cells. p53 is one of the major tumor suppressor proteins in gliomas [15]. Glioma tumors retain wild type p53; hence the ability to maintain the tumor phenotype depends on down regulated expression of p53 [13-15]. Since crocin induces apoptosis so we investigated if the process of apoptosis involves p53 signalling cascade or not. Crocin treatment induced p53 expression in C6 glioma cells compared with the control. Crocin treatment induces reactivation of p53 impeding tumor growth and proliferation of C6 glioma cells. Up-regulation of p53 is believed to be instrumental in cell growth inhibition. Studies have revealed that this pathway is mutated in the majority of gliomas and. is highly regulated by multiple mechanisms, often involving cross-talk with other signaling pathways.

V. CONCLUSION

So concluding crocin has potent anti-tumoral effect on C6 cells based on targeting different processes in in vitro conditions. Crocin induces apoptosis in C6 glioma cells by targeting p53 pathway which is central to number of other processes in gliomaganasis hence inbition of this pathway declines the possibility of drug resistance. Thus crocin can be used as an effective strategy for treating resistant gliomas. More work needs to be done inorder to dealinate the molecular mechanism behind anti glioma effects of crocin.

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