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ORIGINAL ARTICLE

Picrocrocin exhibits growth inhibitory effects against SK-MEL-2 human malignant melanoma cells by targeting JAK/STAT5 signaling pathway, cell cycle arrest and mitochondrial mediated apoptosis

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Summary

Purpose: *Melanoma is one of the lethal types of skin malignancies and is responsible for significant morbidity and mortality across the globe. In this study the anticancer effects of picrocrocin on the human SK-MEL-2 malignant melanoma cell line were evaluated along with its mode of action.*

Methods: *The effect of picrocrocin was evaluated on SK-MEL-2 cells by MTT assay. Apoptosis was determined by DAPI and annexin V/PI staining. The effects on reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and cell cycle analysis were performed by flow cytometry. Expression of the proteins (JAK/STAT5) was estimated by western blotting.*

Results: *Picrocrocin exerted growth inhibitory effects on the SK-MEL-2 melanoma cells. The IC_{50} of picrocrocin against the SK-MEL-2 cells was 20 μM at 24-h incubation. The antiproliferative activity of picrocrocin was due to apoptosis and cell cycle arrest. In addition, picrocrocin enhanced the ROS levels and decreased the MMP levels of SK-MEL-2 cells. Finally, picrocrocin inhibited the JAK/STAT5 signalling pathway in the SK-MEL-2 melanoma cells.*

Conclusions: *Taken together, these results indicate that picrocrocin can prove to be an important molecule in the search of more efficient therapies for melanoma.*

Key words: *anticancer, apoptosis, JAK, melanoma, picrocrocin*

Introduction

Plants exhibit broad diversity in the production of chemical compounds. These metabolites perform a variety of functions in plants and also defend the plants from the harsh environmental conditions [1]. The plant-derived metabolites have also been found to beneficial to humans [2] and have been used as a source of medicines for the treatment of different diseases and disorders. It has been reported that 70% of the global human population are dependent on plants for their basic health care needs [3]. *Crocus sativus (C.Sativus)* is an important medicinal herb that has been used as a spice and medicine since ages [4]. The flowers of this plant are considered important sources of carotenoid-derived compounds. These compounds are known as apocarotenoids and perform many functions in plants [5]. The main apocarotenoids of the flowers of *C. sativus* include crocin, picrocrocin and safranal and have been reported to exhibit a number bioactivities which include anticancer, antiinflammatory and antimicrobial to name a few [6,7]. In this study the anticancer activity of one of

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the saffron apocarotenoids, picrocrocin, was evaluated against human malignant melanoma cells. Previous studies have shown that picrocrocin inhibits the proliferation of several types of cancers. For instance, picrocrocin was reported to inhibit the cell viability of leukemia cells [8]. In this study we report for the first time the growth inhibitory effects of picrocrocin on the human malignant melanoma SK-MEL-2 cell line.

The main objective of the present study was to evaluate the anticancer effects of picrocrocin against SK-MEL-2 cells along with examining its effects on mitochondrial mediated apoptosis, cell cycle phase distribution, reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and JAK/STAT5 signalling pathway. Taken together we conclude that picrocrocin may be utilized in the development of anticancer therapy for the treatment of human malignant melanoma. Hence, further studies for *in vivo* evaluation of picrocrocin and semi-synthesis of more potent derivatives of picrocrocin are required.

Methods

Cell line and culture conditions

Human melanoma cell line SK-MEL-2 was procured from Cancer Research Institute of Beijing, China. The cells were cultured continuously in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS) containing antibiotics (streptomycin 100 µg/ml and penicillin G 100 U/mL) and incubated at 37°C for 24 hrs (5% CO_2 and 95% air).

Antiproliferative assay

The impact of picrocrocin on the viability of SK-MEL-2 cancer cells was investigated by MTT assay. The SK-MEL-2 cells were cultured at a density of 1×10^6 cells per well in 96 well plates for 12 hrs. The cells were subsequently treated with 0-100 µM picrocrocin dose for 24 hrs. Thereafter, MTT solution (20 µL) was added to each well before the addition of 500 µl of dimethyl sulfoxide (DMSO). Formazan crystals were solubilized in DMSO (500 µL). Thereafter, the absorbance was assessed by ELISA plate reader.

Apoptosis assay

SK-MEL-2 cells were seeded in 6-well plates with 1×10^6 cells per well and then they were treated with 0, 10, 20 and 40 µM picrocrocin for 24 hrs. This was immediately followed by DAPI (4',6-diamidino-2-phenylindole) staining. Then, the cell samples were examined and photographed with a fluorescence microscope. To determine the populations of the apoptotic SK-MEL-2 cells, the cells were platted in 6-well plates and subjected to picrocrocin treatment at different doses (0, 10, 20 and 40 µM) for 24 hrs. Afterwards, the cells were harvested and PBS (phosphate buffered saline)-washed. The SK-MEL-2 cells were then treated with Annexin V/FITC and propidium iodide (PI) for 20 min and the apoptotic cell percentage was checked by flow cytometry (BD Biosciences, San Jose, CA, USA).

Analysis of cell cycle phase distribution

To investigate the distribution of the SK-MEL-2 cells in different phases of the cell cycle, approximately 1×10^5 cells in each well of 6-well plates were kept at 37°C overnight to allow the cells to adhere. This was followed by administration of various doses of picrocrocin (0, 10, 20 and 40 µM) and then the cells were incubated at 37°C for 24 hrs. Finally, the distribution of the SK-MEL-2 cells in various cell cycle phases was determined by flow cytometry.

ROS and MMP assays

SK-MEL-2 cells were seeded in 6-well plates at a density of 2×10^5 cells per well, followed by incubation at 37°C for 24 hrs. The cells were then subjected to treatment with 0, 10, 20 and 40µM picrocrocin at 37°C for 24 hrs in 5% CO_2 and 95% air. The cells were then harvested and subjected to PBS washing and resuspension in 500 µL of DCFH-DA (10 µM) for determination of ROS and $DiOC_6$ (1 µmol/l) for assessment of MMP levels at 37°C for 30 min in the dark. Finally, flow cytometry was used for the estimation of ROS and MMP.

Western blotting

The SK-MEL-2 cells were lysed and the protein concentration in each cell lysates was determined by BCA (bicinchoninic acid) assay. The expression of the proteins of interest was determined by western blotting as previously described [9].

Statistics

Data is shown as mean ± standard error of the mean (SEM) and was statistically analyzed using Student's

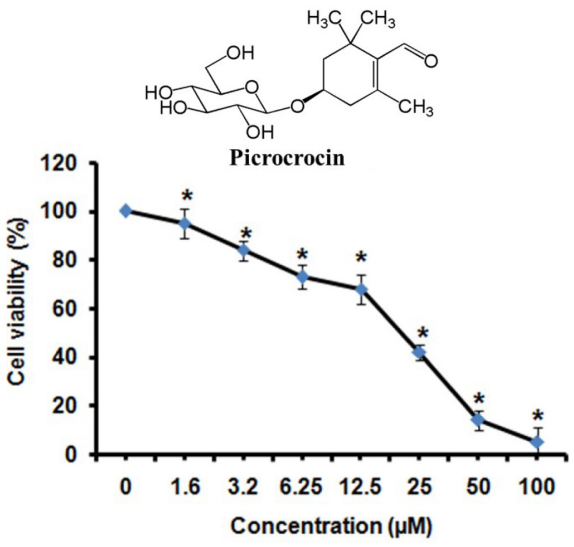

Figure 1. Structure of picrocrocin and its effect on the viability of SK-MEL-2 cells. Experiments were performed in triplicate and values are shown as mean ± SD (*p<0.05).

Newman Keul's test or *t*-test. A p value<0.05 was considered as showing significant difference.

Results

Effects of picrocrocin on SK-MEL-2 cell proliferation

The growth inhibitory effects of picrocrocin on SK-MEL-2 melanoma cell line were evaluated by MTT assay. The SK-MEL-2 cells were subjected to treatment with picrocrocin at varied doses and it was observed that picrocrocin displayed significant anticancer effects against SK-MEL-2 cells with an observed of IC_{50} 20 μM. The effect of picrocrocin on the growth of SK-MEL-2 cells exhibited a concentration-dependent pattern (Figure 1).

Picrocrocin induced apoptosis in SK-MEL-2 cells

The anticancer effects of picrocrocin on SK-MEL-2 cells were subjected to DAPI staining. It was observed that picrocrocin caused apoptosis in SK-MEL-2 cells dose-dependently (Figure 2). The analysis of apoptotic cell population was performed flow cytometrically as depicted in Figure 4. The apoptotic SK-MEL-2 cells increased from 3.2% in control to 56.5 % at 40 μM dose of picrocrocin (Figure 3). To assess whether the apoptosis was due to the activation of mitochondrial apoptotic pathway, the protein expression of Bax and Bcl-2 was examined and the results revealed that the protein expression of Bax increased and of Bcl-2 decreased in a dose-dependent manner (Figure 4).

Picrocrocin activated ROS production and reduction of MMP in SK-MEL-2 cells

The ROS level at different doses of picrocrocin in SK-MEL-2 cells was estimated and the results revealed that the ROS levels of picrocrocin-treated cells enhanced upto 205% at 40 μM picrocrocin (Figure 5). These findings indicated that picrocrocin caused the generation of ROS in SK-MEL-2 cells to trigger apoptosis. ROS generation was related to mitochondrial dysfunction, i.e. disruption of the outer mitochondrial membrane to lead to the discharge of the apoptosis-inducing proteins. Hence, we investigated whether picrocrocin decreased the MMP in SK-MEL-2 cells treated with picrocrocin at different doses. Picrocrocin-treated SK-MEL-2 cells exhibited a considerable reduction in MMP in a dose-dependent manner. The MMP decreased to 44% at 40 μM of picrocrocin (Figure 6).

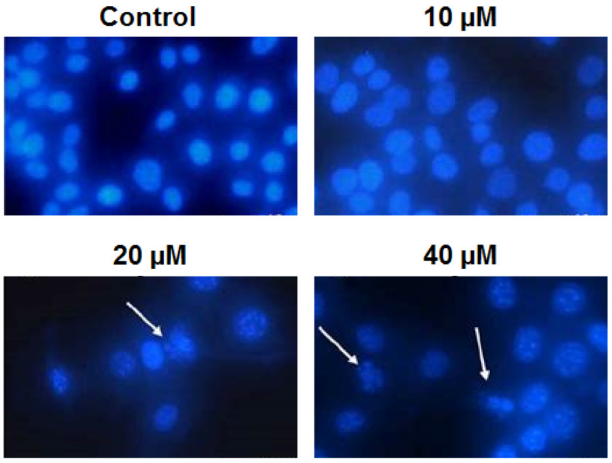

Figure 2. Picrocrocin at various doses induces apoptosis in SK-MEL-2 cells as indicated by DAPI staining. Experiments were performed in triplicate. The arrows indicate the apoptotic cells and it is shown that with increase in dose, the fraction of apoptotic cells increased significantly.

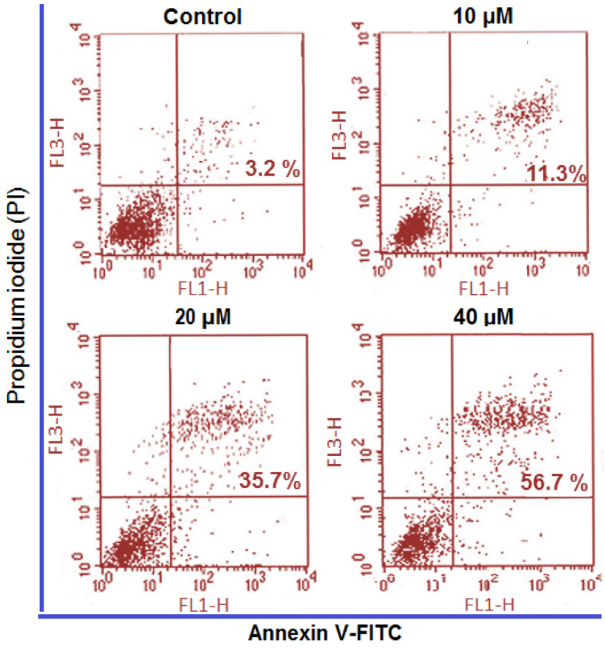

Figure 3. Estimation of the percentage of the apoptotic SK-MEL-2 cells. Experiments were performed in triplicate. In this experiment, annexin V assay was used to quantify the percentage of cells that had undergone early and late apoptosis. The percentage of cells increased greatly with increasing drug dose.

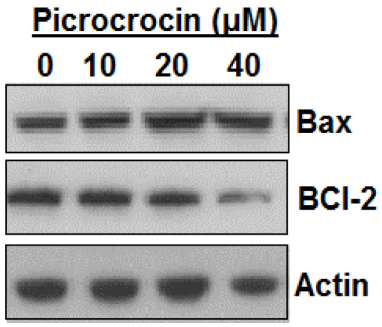

Figure 4. Effect of picrocrocin on the expression of Bax and Bcl-2 proteins as indicated by western blotting. Experiments were performed in triplicate. The Figure shows that the protein expression of Bax increased and of Bcl-2 decreased in a dose-dependent manner.

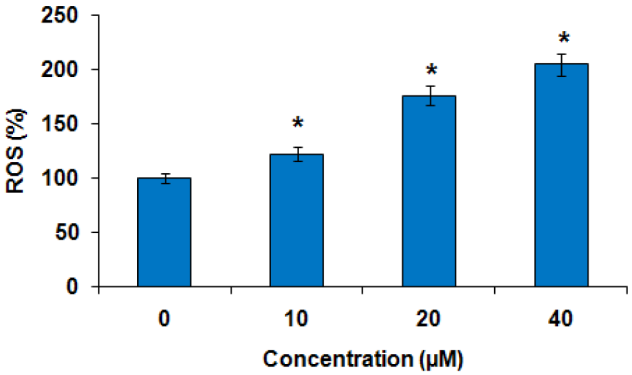

Figure 5. Effect of picrocrocin on the ROS levels in SK-MEL-2 cells. The experiments were performed in triplicate and the results are shown as mean ±SD (*p<0.05). With increase in picrocrocin dose, ROS levels were also increased in a dose-dependent manner.

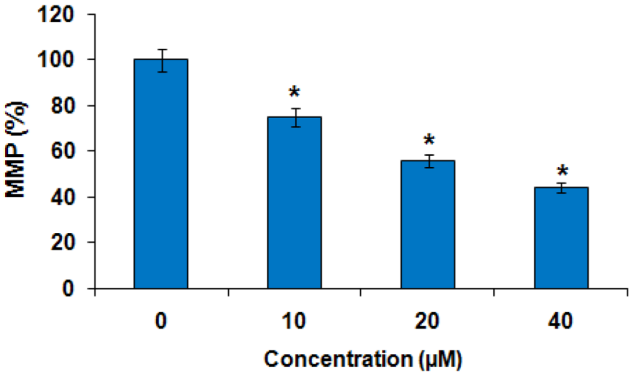

Figure 6. Effect of picrocrocin on the MMP levels in SK-MEL-2 cancer cells. The experiments were performed in triplicate and the results are shown as mean ±SD (*p<0.05). With increase in picrocrocin dose, MMP was also increased in a dose-dependent manner.

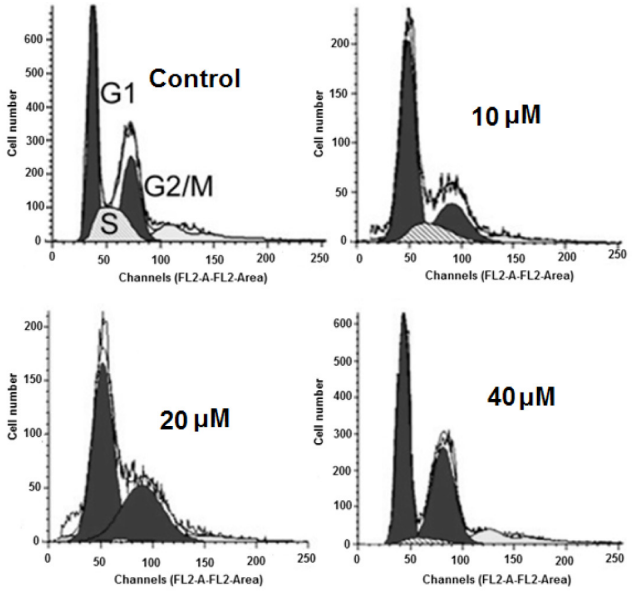

Figure 7. Picrocrocin triggered G2/M cell cycle arrest in SK-MEL-2 cells as shown in the flow cytometric analysis. Experiments were performed in triplicate.

Picrocrocin triggered cell cycle arrest

To investigate the impact of picrocrocin on the distribution of the SK-MEL-2 in different phases of the cell cycle, the cells were treated with 0, 10, 20 and 40 μM of picrocrocin for 24 hrs and the results revealed that the percentage of cells in G2 phase increased in a dose-dependent manner causing cell cycle arrest (Figure 7).

Picrocrocin inhibited the JAK/STAT5 signalling pathway

The effects of picrocrocin were also evaluated on JAK/STAT5 signalling pathway. The results showed that picrocrocin could inhibit the phosphorylation of p-JAK1/2 and p-STAT5. However, the expression of JAK1/2 and STAT-5 remained unaltered (Figure 8).

Discussion

Melanomas arise from melanocytes (the pigment-forming cells) or in some cases from the precursors of melanocytes in the skin. Human melanoma is reported to be one of the lethal types of skin malignancies [10]. Melanomas lead to about 50,000 deaths annually worldwide [11]. The treatment of melanoma generally depends on the stage at which it is diagnosed. If diagnosed early, surgical interventions are carried out to cure the patients completely. However, at advanced stages these malignancies are generally difficult to treat

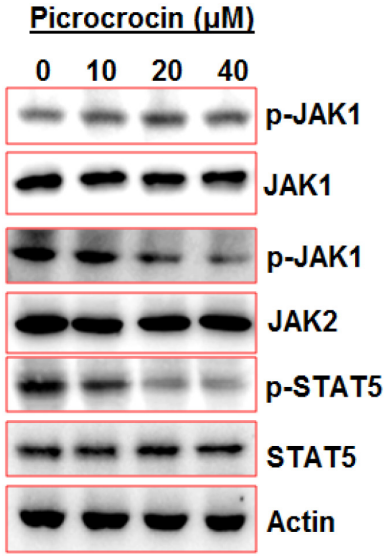

Figure 8. Effect of picrocrocin on the JAK/STAT5 pathway as indicated by western blotting. The Figure shows that picrocrocin could inhibit the phosphorylation of p-JAK1/2 and p-STAT5. However, the expression of JAK1/2 and STAT-5 remained unaltered. The experiments were performed in triplicate.

and require multi-disciplinary approaches and the results are often far from descent [12]. Moreover, the chemotherapy currently used creates many adverse effects and its results are not satisfactory. Therefore, several molecules are evaluated against the malignant melanoma cells. In this study we evaluated an important apocarotenoid of saffron, picrocrocin, against the malignant melanoma SK-MEL-2 cells and the results indicated that this compound could exert dose-dependent antiproliferative effects on these cells. These results are also well supported by previous investigations wherein saffron apocarotenoids have been reported to inhibit the growth of several types of cancer cells. For instance, crocin apocarotenoid has been reported to inhibit the growth of cervical cancer cells [13]. In yet another study, saffron extracts have been reported to inhibit the growth of breast cancer cells [14]. Similarly, crocetin apocarotenoid has been reported to inhibit the proliferation of breast cancer cells [15]. Saffron apocarotenoids, such as crocin, have been found to inhibit the proliferation of gastric adenocarcinoma cells by inducing apoptosis and modulation of Bax/Bcl-2 ratio [16]. Apoptosis is one of the conserved processes that helps in the removal of the harmful and unwanted cells from the body [17]. In this study picrocrocin was found to induce apoptosis in the human melanoma MSK-MEL-2 cells which was associated with alteration in the expression of Bax/Bcl-2 ratio. Furthermore, picrocrocin could also enhance ROS and reduce MMP levels in the SK-MEL-2 cells indicating that picrocrocin might be inducing apoptosis via the mitochondrial pathway. Saffron apocarotenoids have also been reported to trigger cell cycle arrest. In this study we observed that picrocrocin triggers G2/M cell cycle arrest in the SK-MEL-2 melanoma cells. JAK/STAT pathway is one of the important pathways by which anticancer agents exert their effects on the cancer cells [18]. In this study it was observed that picrocrocin inhibited the expression of p-JAK1, p-JAK2 and STAT5, indicative of the potential of picrocrocin as an anticancer agent.

Conclusion

In conclusion, picrocrocin inhibited the proliferation of human malignant melanoma cells. Its antiproliferative effects were due to the induction of apoptosis and cell cycle arrest. Besides, picrocrocin could also inhibit the JAK/STAT5 signalling pathway in human melanoma cells. Taken together, picrocrocin can prove to be an important lead molecule for the treatment of melanoma.

Conflict of interests

The authors declare no conflict of interests.

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