The effects of crocetin, extracted from saffron, in chemotherapy against the incidence of multiple drug resistance phenotype

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Keywords: Crocetin, one of the main substances of saffron extract, has anti-cancer effects. Drug resistance proteins (e.g. MRP1 and MRP2) are important reasons for the failure of cancer therapy. We intended to investigate the efficacy of crocetin on MRP, and MRP, activity in human ovarian cisplatin-resistant carcinoma cell line (A2780-RCIS).

Materials and Methods: The cytotoxic effect of crocetin was evaluated by the MTT assay. The efficacy of crocetin on MRP and MRP expression at mRNA level was studied by real-time RT-PCR. The effect of crocetin on the activity of MRP transporters was determined by drug efflux assay.

Results: Crocetin decreased cell proliferation in the A2780 (IC50: 183±7 µM) and A2780-RCIS (IC50: 316±9 µM). Crocetin decreased the expression level of MRP1 (22±2 %) and MRP2 (48±8 %) in A2780-RCIS and inhibited MRP pumps function directly in A2780 (44±1 %) and A2780-RCIS (88±10 %) and indirectly in A2780 (32±2 %) and A2780-RCIS (48±15 %) respectively.

Conclusion: Our findings showed that crocetin could quench drug resistance through modulation of MRP transporters in the drug resistant human ovarian cancer cells.

Introduction

The enormous family of cancer diseases consists of abnormal cell growth correlated by the probability to attack or outspread to other sections of the body. In each year, cancers cause of about 9 million deaths that is about 16 % of human deaths worldwide (1). The major methods of cancer treatment include chemotherapy, radiation therapy, and surgery. Chemotherapy is known as one of the most effective cures for metastatic tumors. Chemotherapy is done as monotherapy or combination therapy, which has more beneficial effects in metastatic cases (2). Although chemotherapy is effective in early phases of cancer spreading, but in more advanced stages of cancer, cancer cells usually resist to chemotherapy agents. The simultaneous resistance of cancer cells to multiple drugs, without any structural or functional correlation (resistance to multiple drugs), is still one of the primary barriers for successful chemotherapy (3).

The reason of cellular resistance to anticancer drugs are divided into two general categories including the factors that affect the access of cancer cells to anticancer agents, and genetic and epigenetic changes in cancer cells that affect their drug sensitivity.

Multiple drug resistance (MDR) is one of the main reasons for unsuccessful cancer chemotherapy. Over-expression of the membrane transporter proteins cause generic MDR that ase low concentration of chemotherapeutic agents by throwing them out of cells (4). These pharmaceutical efflux proteins are part of the ATP-binding cassette transporters family that called ABC family (5). Due to its widespread distribution in the body, ABC proteins play an critical role in the transfer of various types of endogenous substrates. One of the major classes of the ABC family are MRPs (the multiple drug resistance associated proteins) that contains seven members (6).

In the last decades, several researches introduced new herbal compounds with possible anti-neoplastic effects that has created a hope to expanding safer and more operative anti-cancer treatments (7). *Crocus sativus* (saffron) from *Iridaceae* family has been used as a food additive for years (8). Most of the therapeutic effects of saffron is due to safranal, picrocrocin and crocin (9). Crocin, as one of major carotenoids of saffron, has displayed anti tumoral effects both in *in vitro* and *in vivo*. Crocin could affect the action of cellular proteins, but the exact target of crocin and other carotenoids of the saffron have not been recognized yet (10). It has been previously showed that crocin has inhibition effect on MRP1 and MRP2 transporters (11). Crocetin, a natural apocarotenoid dicarboxylic acid, is the central core of crocin (12). It showed cytotoxic effects in various cellular models and on different kinds of tumors including leukemia, ovarian and breast carcinoma.
Materials and Methods

Materials

Crocetin was extracted from saffron using the procedure represented in the Iran patent no. 84459. In order to determine the purity of crocetin, the crocetin absorption spectrum in dimethyl sulfoxide (DMSO) was detected in the ultraviolet light region at wavelengths of 200 to 700 nm. Two distinct peaks at wavelengths of 436 and 464 nm were found to be consistent with the pure crocetin absorption spectra.

Ovarian cancer cell lines (A2780 and its drug resistant A2780-RCIS derivative that overexpress MRPs) were kindly supplied by Professor Herman Lage (Molecular Pathology Department, Charite Campus Mitte, Berlin, Germany). In order to preserve the cisplatin-resistant phenotype of A2780-RCIS cells, the culture medium was supplemented with cisplatin 33.3 µM and kept at −20 °C.

Preparation of the crocetin solution

For each experiment, crocetin was dissolved in DMSO to a final concentration of 60 mM and kept at −20 °C. Crocetin was diluted by complete culture medium to its final concentration (25, 50, 100, 200 and 400 µM), exactly before each experiment. The maximum concentration of DMSO in 400 µM crocetin solution was about 0.65 % (v/v) while the safest range of DMSO is up to 0.5% (v/v) (15). Therefor, the cytotoxic effects of DMSO on tested cell lines were evaluated by MTT assay.

Cell culture

Cells were routinely cultured in RPMI-1640 containing FBS and penicillin/streptomycin and stored at 37 °C in CO₂ 5%. This study was approved in the Research Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran.

MTT cytotoxicity assay

The cytotoxic effects of crocetin on the tested cell lines, were determined by MTT cytotoxicity assay. Ten thousand cells were seeded in 96-well plates and treated with various concentrations of crocetin (0-400 µM). The control wells had DMSO at equivalent concentrations to those used for the test mixtures. Cell viability was evaluated after 48 hr by adding MTT in PBS solution (0.5 mg/ml) to each well for 4 hr. Finally, the crystals of formazan were dissolved in 200 µl of DMSO and absorbance was assessed by an ELISA plate reader (BioTek, Germany) at 550 and 630 nm (test and reference wavelength, respectively). The ratio of ODtest/ODcontrol × 100 was reported as the percentage of cell viability and the IC₅₀ values were intended as the concentration of crocetin that decreased the cell viability to half.

Real-Time RT-PCR

The MRPl and MRPr mRNA expression of drug resistant cells (A2780-RCIS) was evaluated by real-time RT-PCR after treating cells with various concentrations of crocetin (0-200 µM) for 48 hr. Total cellular RNA of 5 × 10⁵ cells was extracted by Parstoos RNA isolation kit. The total RNA concentration and the quality of them was measured by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and the acceptable range A₂₆₀/A₂₃₀ and A₂₆₀/A₂₈₀ ratios was among 1.8 to 2.2. cDNA was synthesized using Parstoos cDNA kit using 300 ng of total RNA. Real-time RT-PCR tests were performed by the Parstoos SYBR green master mix Kit and real-time cycler step-one PLUS ABI (ABI, USA). The sequences of primers were: MRPl, 5’-TGTTTTCTTGTCGACCAACT-3’ (forward) and 5’-TTGGATCTCAGGATGGCTAGG-3’ (reverse) (11); MRPr, 5’-AGCAAGCATAGAGCTGGCCCTT-3’ (forward) and 5’-AGGAGGAGCAATGATCTTGATCTGTG-3’ (reverse) (16); β-actin: 5’-TCATGAAATGTGACGTCGACATC-3’ (forward) and 5’-AGAAAACCAAGAGCCATAGTGCC-3’ (reverse) (17). Reactions were carried out with an initial activation step at 94 °C for 15 min followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min as PCR amplification cycles. The specificity of primers was evaluated by melting curve analysis. The expression levels of MRPl or MRPr were normalized to the β-actin by the step-one PLUS system. The comparative expression level of tested genes were informed as the following equation: (target/reference ratio of the treated cells)/ (target/reference ratio of the untreated control cells) (11).

Efflux assay

Doxorubicin and furosemide are known to be MRPl, substrate and MRPl pump inhibitor, respectively (18, 19). In this study we aimed to find out whether crocetin can inhibit the efflux of doxorubicin from the A2780 and A2780-RCIS cells. The direct and indirect effects of crocetin on the inhibition of function of MRPl transporters were evaluated by efflux assay. For evaluation of direct intraction of crocetin with MRPl transporters, A2780 cell lines were exposed to crocetin (0-200 µM) and 10 µM doxorubicin in the presence or absence of furosemide (1 mM) (19) for 1 hr. After that, the medium was shift to the fresh culture medium in the presence or absence of furosemide (1 mM) for 3 hr. Then the supernatants were transferred to the black 96-well plates and doxorubicin
content was assayed spectrofluorimetrically at 470 and 585 nm (excitation and emission wavelength, respectively). For evaluation the indirect effects of crocetin on the function of MRP transporters (by changing their expression), all cells were exposed to crocetin (0-200 μM) for 48 hr. Then, the supernatants were replaced with 10 μM doxorubicin in the presence or absence of furosemide (1 mM) for 1 hr. Finally, the supernatants were replaced with fresh culture medium with or without furosemide (1 mM) for 3 hr and continued protocol as described in the previous section. Performance level of drug resistance transporter was evaluated by percent of doxorubicin efflux by using the following ratio: ((Fluorescence amount in the absence furosemide in the sample-Fluorescence amount in the presence of furosemide in the sample)÷(Fluorescence amount in the absence of furosemide in the control sample-Fluorescence amount in the presence of furosemide in the control sample))× 100 (20).

Statistical analysis

Data (mean±SEM) were reported from three independent experiments. Statistical analyses were performed by SPSS version 16.0 using ANOVA, with the Tukey’s post-hoc test. A value of P<0.05 was considered statistically significant.

Results

Cytotoxicity assay

To examine the effects of crocetin on the ovarian cancer cells viability, all cells were incubated with various concentrations of crocetin (0-400 μM) at 37 °C for 48 hr. Cell viability was evaluated by the MTT assay and compared with control. Each experiment was repeated independently three times in triplicate tests and data are shown as mean ± SEM. ***P≤0.001

Figure 1. The effects of crocetin on cell viability of A2780 (A) and A2780-RCIS (B) cell lines. The cells were incubated with different concentrations of crocetin (0-400 μM) at 37 °C for 48 hr. Cell viability was evaluated by the MTT assay and compared with control. Each experiment was repeated independently three times in triplicate tests and data are shown as mean ± SEM. ***P≤0.001

The effect of crocetin on the gene expression of MRP1 and MRP2

In order to assess the effects of crocetin on the expression of MRP1 and MRP2 at mRNA level, the resistant cells (A2780-RCIS) were treated with various concentrations of crocetin (0-200 μM) for 48 hr. Real-time RT-PCR results displayed that crocetin can reduce the expression levels of MRP mRNA in a concentration-independent manner. Maximum inhibition was statistically significant at 25 μM for MRP1 mRNA (up to 22 ± 2%) (Figure 2a) and 200 μM for MRP2 mRNA (up to 48 ± 8%) (Figure 2b) in A2780-RCIS cells.

Effect of crocetin on MRP1 and MRP2 efflux activity

Exposure of A2780 cell line to all concentrations (0-200 μM) of crocetin decreased doxorubicin efflux in both direct (up to 44±1 % at 100 μM) (Figure 3a) and indirect (up to 32±2 % at 100 μM) (Figure 3b) efflux assay. Interestingly in A2780-RCIS cell line, direct suppression of MRP transporters was statistically significant in all
Crocetin against the incidence of MDR

Discussion

Cancer is still one of the most challenging diseases. Chemotherapy is the most common way to treat cancer, but its effectiveness can be reduced by multi-drug resistance (MDR). One of the major mechanisms in MDR is the presence of drug transporters that pump chemotherapeutic agents out of the cell (21). Crocetin is a derivative of crocin, one of the major components of saffron extract. Recent studies have shown that crocetin exhibits many anti-cancer properties, including suppression of nucleic acid synthesis and induction of apoptosis (22). In this study, we assessed the effects of crocetin on the function and expression of MRP1 and MRP2 transporters in the human ovarian carcinoma cell line.

It has been formerly displayed that MRP2 over-expression in human cancer cell lines raise the resistance to doxorubicin (23), while inhibition of MRP2 expression improve cell sensitivity to doxorubicin (24). According to these studies, doxorubicin has been considered as a substrate of MRP1 and MRP2 pumps in parent cells in comparison to resistant cells. Although the cytotoxicity of DMSO is high in parent cells but the effect of crocetin compared to DMSO is statistically significant. Also, in order to prevention of undesired effect of DMSO on the results of this study, lower concentration than 200 µM have been used for other tests.

In this research, we proposed to assess the effects of crocetin on MRP transporters expression and function. The MRP activity evaluation has been performed in two ways, short term disposal with crocetin to check the direct interaction between crocetin and existing active MRP transporters, and long term disposal with crocetin to check the indirect effect of crocetin on the activity of MRP transporters as a result of its regulations on their expression level. Interestingly, crocetin could...
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Abstract

Decrease doxorubicin efflux after 1 and 48 hr in both drug-resistant A2780-RCIS cell line and A2780 parent cell. But the direct inhibition effect was more efficient than indirect inhibition effect in both resistant and sensitive cells. Then again, the real-time RT-PCR findings displayed that crocetin meaningfully decreased the MRP1 and MRP2 mRNA expression level at only two concentrations (25 µM for MRP1 and 25 and 200 µM for MRP2) in A2780-RCIS cells. Our results showed that the main path of MRP transporters inhibition by crocetin is directly by inhibition of efflux activity of these two transporters and the reduction of MRP gene expression may not be the major path for increasing doxorubicin cytotoxic effects on cisplatin resistant ovarian carcinoma cells.

It has been previously showed that some carotenoids are competitive inhibitors of ABC-transporters (25, 26). They (crocin, β-carotene, canthaxanthin, retinoic acid, and fucoxanthin) can utilize MDR reversal and increase the cytotoxic effect of chemotherapeutic agents in human CEM/ADR5000 and Caco-2 cells (27). Also it has been shown that crocetin induces cytotoxic effect and increases vincristine-induced cancer cell death in MCF-7/VCR (the vincristine-resistant breast cancer cell line) and it can be used as a chemosensitizer for vincristine (28). Entirely, these findings proposed that simultaneous use of crocetin with chemotherapeutic agents in cancer treatment might be an efficient method to increase the effectiveness of chemotherapy and modulate the influence of MDR.

Conclusion

In this study we intended to figure out the capability of crocetin on suppressing MDR in human ovarian cancer cells by inhibiting MRP1 and MRP2 transporters. The outcomes showed that crocetin could reduce the MRP efflux function and perform as MDR reversal agent that increase the cytotoxic effects of doxorubicin in the A2780-RCIS. This study proposed that the usage of crocetin in combination with chemotherapeutic drugs in cancer therapy could be an effective way to increase the efficacy of chemotherapeutics and limiting the impact of MDR transporters.

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Conflicts of Interest

The authors declare that they have no competing interests.

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