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Crocin reversed the antitumor effects through up-regulation of MicroRNA-181a in cervical cancer cells

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Abstract

Cervical cancer ranks third in cancer incidence worldwide and is the most frequent gynecological cancer in developing countries. This study aimed to investigate the effect of crocin on the resistance of HELA/Ara-C cells to the chemo-agent of Ara-C. HELA/Ara-C cells which was defined as Ara-C-resistant HELA cell line, were treated with crocin. After that, cell proliferation, apoptosis, and cell cycle assay were examined by MTT assay, AO/EB staining, and flow cytometry, respectively. In this study, we found that crocin combining with Ara-C significantly inhibited the proliferation HELA/Ara-C cell, and this inhibitory effect was time dependent. Moreover, crocin combining with Ara-C could induced apoptosis and cell cycle arrest of HELA/Ara-C Cells. Further then, HELA/Ara-C cells were transfected with the mature miRNA-181s mimic and the control miRNA mimic, respectively. Quantitative RT-PCR results demonstrated that compared with HELA/Ara-C Cells +Ara-C group, miRNA-181a was upregulated 1.5-fold in HELA/Ara-C Cells + crocin group and 1.7-fold in HELA/Ara-C Cells and crocin +Ara-C group, respectively. The other miRNA-181s showed no significant differences. In summary, this was the first study to show that miRNA-181a might be involved in the development of crocin induced reversing Ara-C resistance in HELA/Ara-C cell lines.

Keywords: MiRNAs; crocin; HELA/Ara-C cells.

Practical Application: MiRNA-181a could be used in the development of crocin induced reversing Ara-C resistance in HELA/Ara-C cell lines.

1 Introduction

Cervical cancer ranks third in cancer incidence worldwide and is the most frequent gynecological cancer in developing countries (Little et al., 2018; Gutierrez & Kentsis, 2018). The frequency of cervical cancer after treatment for dysplasia is lower than 1% and mortality is less than 0.5% (Maruffi et al., 2018). The increasing trend of the disease in developing countries is attributed to the early beginning of sexual activities, certain sexual behaviors like high number of multiple partners, early age at first intercourse, infrequent use of condoms, multiple pregnancies with Chlamydia association, and immunosuppression with HIV, which is related to higher risk of HPV infection (Maruffi et al., 2018; Buckley et al., 2018; Watts & Tallman, 2014; Rafiq et al., 2020).

Ara-C is an effective agent in the treatment of cervical cancer (Murphy & Yee, 2017; Funato et al., 2000; Grant, 1997). It is a nucleoside analogue antimetabolite, which exerts its function through its derivative Ara-CTP. Ara-CTP incorporates into DNA, which induces DNA double-strand breaks and subsequent cell death. Although Ara-C-based chemotherapeutic treatments achieve dramatic remissions, the majority of patients relapse with drug-resistant disease. The reasons underlying the drug resistance to Ara-C attract extensive laboratory investigations. The deoxycytidine kinase (dCK) has been believed to be one of the mechanisms of Ara-C resistance (Bista et al., 2017; Kurata et al., 2016; Kanno et al., 2007; Eor et al., 2021).

MicroRNAs (miRNAs) are the most stable nucleic acid molecules in the body and only about 19 to 23 nucleotides acted ad regulating gene expression at the post-transcriptional level through complex miRNA-mRNA interactions (Naidu et al., 2015). Increasing findings have shown that microRNAs are involved in tumor cells' resistance to chemotherapeutic agents (Ozawa et al., 2018; Meghani et al., 2018; Geretto et al., 2017; Gaspar-Pintiliescu et al., 2020). But the role of microRNAs in drug resistance to Ara-C in AML is largely unknown.

Crocus sativus L., commonly known as saffron, is a perennial stemless herb of the large Iridaceae family and has been used in cancer therapy (Colapietro et al., 2019; Wang et al., 2018; Hire et al., 2017). It is a main water-soluble carotenoid of the saffron extract, exhibiting anti-tumor activity against many human tumors, such as colorectal, pancreatic, and bladder cancer. Notably, crocin significantly inhibits the growth of cancer cells but has no effects on normal cells (Shi et al., 2018; Gu et al., 2018; Amerizadeh et al., 2018). Our previous study showed that crocin inhibited the proliferation and tumorigenicity of HELA cells, which may be mediated by the induction of apoptosis and cell cycle arrest and the regulation of Bcl-2 and Bax expression (Sun et al., 2013; Balthazar et al., 2021). In present study, we focused on HELA/Ara-C cells and aimed to further investigate the effect and the potential mechanism of crocin on the Ara-C resistance of HELA/Ara-C cells. These results from this study would supply innovations to develop new drugs with high efficacy and low toxicity for the treatment of cervical cancer.

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2 Materials and methods

2.1 Cell lines and culture conditions

Cell Line and Treatment. Human cervical cancer HELA cells and HELA/Ara-C cells, which was defined as Ara-C-resistant HELA cell line. HELA cells and HELA/Ara-C cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified incubator of 5% CO2 at 37 °C. Crocin was purchased from Sigma (CAS Number 42553-651) and diluted in 10 mmol/L phosphate-buffered saline for the appropriate concentration upon used.

2.2 Exogenous overexpression of the mature miRNA-181s through transient transfection of the mature miRNA-181s mimic

The mature miRNA-181s (miRNA-181a, miRNA-181b and miRNA-181c) mimic and control miRNA mimic was chemically synthesized by Shanghai GenePharma Company. The sequence of the mature miRNA-181s mimic and control miRNA mimic is shown in Table 1. HELA/Ara-C cells were plated in 6-well plates (6% 105 cells/well) and transfected with 100 nM of the mature miRNA-181s mimic or 100 nM control miRNA mimic using Lipofectamine 2000 (Invitrogen, Long Island, NY) according to the manufacturer's protocol.

2.3 Cell proliferation assay

Cell proliferation was determined by using MTT assay. Briefly, HELA/Ara-C cells were treated with crocin (5 mg/mL) for 24 h or 48 h. Then the cells were incubated with MTT solution (5 mg/mL in PBS, Sigma) for 4 h and solubilized with DMSO (150 μL). The absorption was measured at 570 nm in an ELISA reader. The following formula was used for the calculation: cell inhibition rate (%) = 1 – (*A* value of the experimental samples/*A* value of the control) × 100%.

2.4 Cell cycle analysis

HELA/Ara-C cells were treated with different concentrations of crocin. After 48 h, cells were harvested and fixed in 70% ethanol at 4 ∘C overnight. Fixed cells were stained with 5 μL PI for 20 min on ice in the dark. Finally, the fluorescence emitted by PI-DNA complex was examined at 488 nm. The percentages of cells in various phases of the cell cycle, namely, G0, G1, S, and G2/M, were assessed using a flow cytometry and analyzed by Cell Quest software.

2.5 Apoptosis assay

Flow cytometry was used to detect apoptosis of the transfected HELA/Ara-C cells by determining the relative amount of AnnexinV-FITC-positive, PI-negative cells as previously described, respectively.

2.6 In vitro drug sensitivity assay

HELA/Ara-C cells were seeded into 96-well plates in RPMI-1640 medium containing 10% FBS. After 24 h, the cells were treated with serial dilutions of Ara-C. In addition, as to HELA cells, 24 h after transfection of the mature miRNA-181s mimic or control miRNA mimic, cells were also seeded into 96-well plates for next step experimentation. freshly prepared anticancer drug Ara-C was added with final concentration being 0.01, 0.1, 1 and 10 uM of the human peak plasma concentration for each drug as previously described. The peak serum concentrations of various anticancer drugs are 0.4 mg/mL for Ara-C. Forty-eight hours after addition of drugs, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazo-lium bromide (MTT) assay.

2.7 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The amplification of miRNA was performed using the specific primers of reverse transcription (RT) and polymerase chain reaction (PCR) from Bulge-Loop™ miRNA qRT-PCR Primer Set (RiboBio, Guangzhou, China) as previously described. The quantification of PCR product was evaluated by the level of fluorescence in emitted by SYBR Green (SYBR® Premix Ex Taq™ II, TaKaRa). RT and PCR were performed as previously described (Zhao et al., 2013; Du et al., 2016). RT reactions were carried out at 42 °C for 60 min followed by 70 °C for 10 min. The qRT-PCR was run on a LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) in 384-well plates at 95 °C for 20 s followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s and then 70 °C for 10 s. The melting analysis was added finally to evaluate the specificity of PCR products. expression of miRNAs was calculated based on comparative 2-ΔΔCt method and let-7a was used as internal control (Li et al., 2015a, b). The raw data were presented as the relative quantity of target miRNA, normalized with respect to let-7a and compared with a reference sample. The primer of miR-181a and let-7a were as follows: miR-181a: forward: 5'-AGATCTAGCCCAATATCGGCCATGTT-3' and reverse: 5'- CTCGAGAGAAAGTCCTGGTGTGTCCA-3'; let-7a: 5'-GCCGCTGAGGTAGTAGGTTGTA-3' and reverse: 5'-GTGCAGGGTCCGAGGT-3'.

Table 1. The sequence of the control miRNA mimic and the mature miR-181s mimic.

miRNA mimics			Sequence
has-miR control mimic	5' to 3'	Sense	UUCUCCGAACGUGUCACGUTT
		Antisense	ACGUGACACGUUCGGAGAATT
has-miR-181a mimic	5' to 3'	Sense	AACAUUCAACGCUGUCGGUGAGU
		Antisense	UCACCGACAGCGUUGAAUGUUUU
has-miR-181b mimic	5' to 3'	Sense	AACAUUCAUUGCUGUCGGUGGGU
		Antisense	CCACCGACAGCAAUGAAUGUUUU
has-miR-181c mimic	5' to 3'	Sense	AACAUUCAACCUGUCGGUGAGU
		Antisense	UCACCGACAGGUUGAAUGUUUU

2.8 Statistical analysis

Continuous variables were expressed as mean ± SD (standard deviation) and compared using a two-tailed unpaired Student's t test. Statistical analyses were conducted with the SPSS for Windows version 18.0 release (SPSS, Inc., Chicago, IL). A value of $P < 0.05$ was considered significant in all the analysis.

3 Results

3.1 Crocin combined with Ara-C inhibits the proliferation of HELA/Ara-C cells

MTT assay showed that compared with the control group and solely crocin group, crocin combined with Ara-C have reversed the resistible effect of HELA/Ara-C Cells to the chemical agent of Ara-C. Based on our previous result, crocin showed maximum biological effect at the concentration of 5 mg/mL. After combining with Ara-C (0.4 mg/mL), they demonstrated significantly inhibiting effect on HELA/Ara-C cell proliferation, and the inhibitory effect of crocin combined with Ara-C on HELA/Ara-C cells proliferation was time dependent (Figure 1).

3.2 Crocin combined with Ara-C induced apoptosis and cell cycle arrest of HELA/Ara-C cells

We further performed cell cycle and cell apoptosis analysis to determine whether crocin combined with Ara-C inhibits the proliferation of HELA/Ara-C cells through the regulation of cell cycle progression and apoptosis. In HELA/Ara-C cells, we observed that crocin slightly increased the apoptosis compared with the control group. However, compared with the control and solely crocin groups, a remarkable increasing in apoptosis, as assessed by flow cytometry, was observed after crocin combined with Ara-C treatment, (Figure 2A and 2C). Meanwhile, we performed flow cytometry using PI staining. We observed a significant increase of G0/G1 cells from 21.3% in control group to 42.5% in the crocin-treated group (5.0 mg/mL). Furthermore, significant increase of G0/G1 cells from 42.5% in crocin-treated (5.0 mg/mL) group to 73.6% in the crocin combined with Ara-C treatment group (Figure 2B). These results suggested that crocin combined with Ara-C could induce HELA/Ara-C Cells apoptosis.

3.3 miRNA-181a is downregulated in HELA/Ara-C cells compared with HELA cells

Previous reports showed that miRNA-181s were associated with multiple drugs resistance. In this study, we compared the miRNA-181s in HELA/Ara-C Cells and HELA Cells. Quantitative RT-PCR results demonstrated that miRNA-181a was downregulated 2.2-fold in HELA/Ara-C cells compared with HELA cells (Figure. 3A).

3.4 miRNA-181a modulates Ara-C resistance of HELA/Ara-C cell lines

To investigate whether miRNA-181a had a direct function in Ara-C resistance development or is just differentially modulated in drug resistible cancer cells, we transfected HELA/Ara-C cells with the mature miRNA-181s mimic and the control miRNA mimic, respectively, to observe the effects on drug resistance phenotype thereafter. In HELA/Ara-C cells, MTT assay revealed that only those transfected with miRNA-181a mimic exhibited greatly enhanced sensitivity to Ara-C, compared with those transfected with the control miRNA mimic, as indicated by significantly decreased IC50 values (Figure 3B). Furthermore, after adding with crocin, miRNA-181a mimic group exhibited greatly enhanced sensitivity to Ara-C, compared with those transfected with the control miRNA mimic. In particularly, the enhanced sensitivity to Ara-C was significant higher in crocin plus Ara-C group than that of Ara-C group (Figure 3B).

3.5 Crocin reversed the antitumor effects of HELA/Ara-C cells through UP-regulation of MicroRNA-181a

To further determine the effect of crocin on HELA/Ara-C cells, we compared the miRNA-181s among the three groups including HELA/Ara-C Cells group, HELA/Ara-C Cells plus crocin group and HELA/Ara-C Cells plus crocin plus Ara-C group. Quantitative RT-PCR results demonstrated that miRNA-181a was upregulated 1.5-fold in HELA/Ara-C Cells plus crocin group and 1.7-fold in HELA/Ara-C Cells plus crocin plus Ara-C group, respectively, compared with HELA/Ara-C Cells (Figure 3C). The other miRNA-181s showed no significant differences.

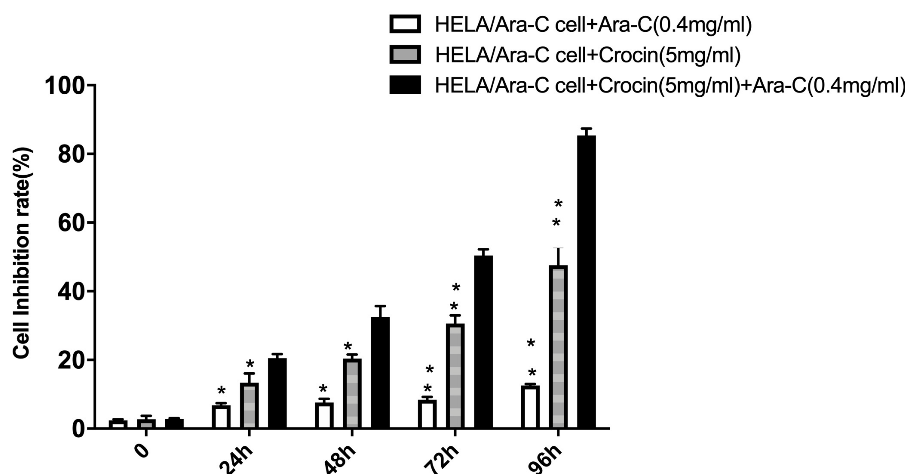

Figure 1. Crocin combined with Ara-C inhibits the proliferation of HELA/Ara-C cells. *p<0.05, ** p<0.01.

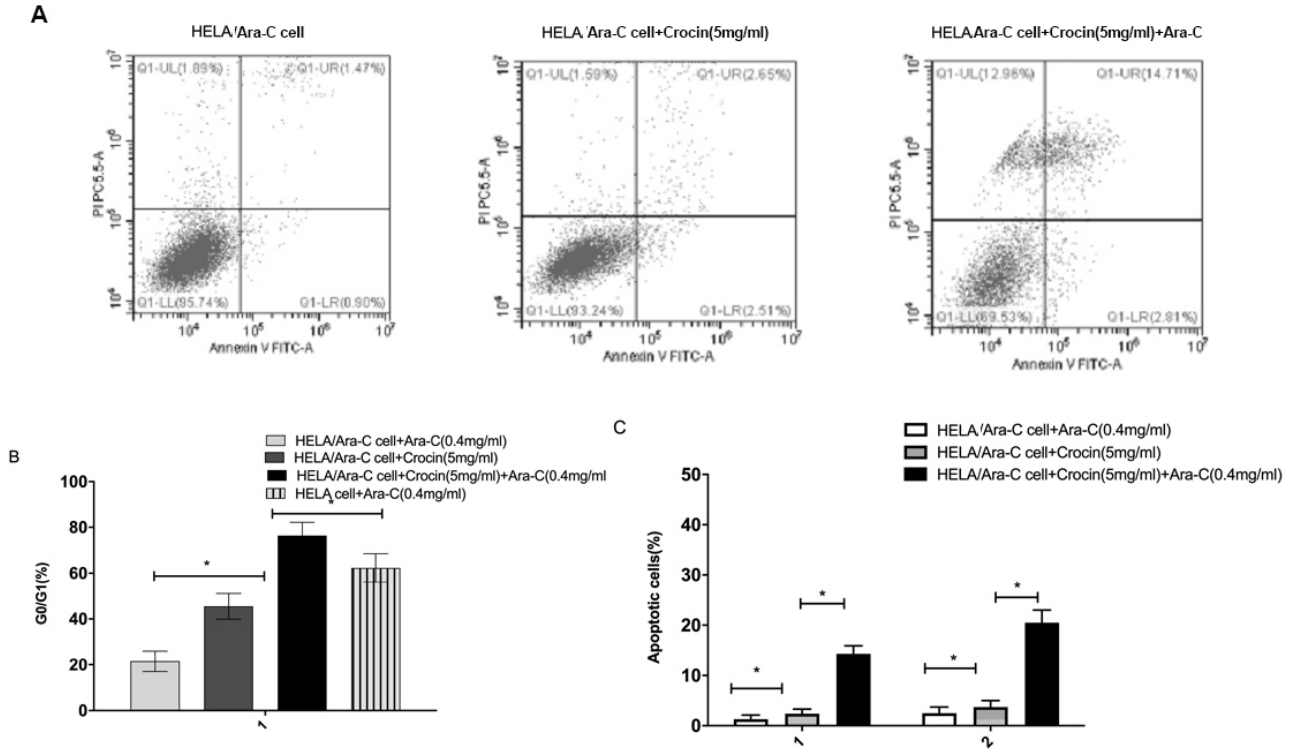

Figure 2. Crocin combined with Ara-C induced apoptosis and cell cycle arrest of HELA/Ara-C cells. A/C: Crocin combined with Ara-C treatment increased the apoptosis compared with the control group; B: significant increase of G0/G1 cells from 42.5% in crocin-treated (5.0 mg/mL) group to 73.6% in the crocin combined with Ara-C treatment group. $^{*}p<0.05$.

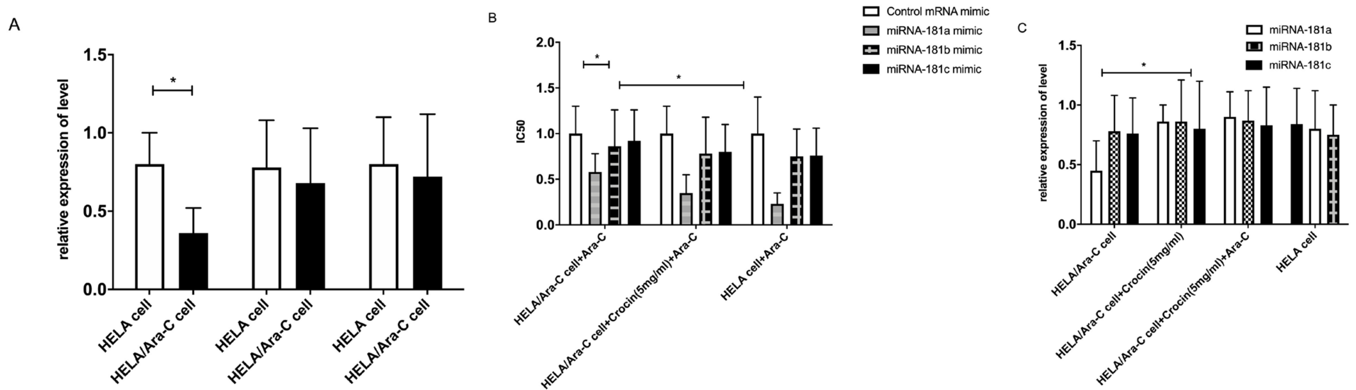

Figure 3. A: quantitative RT-PCR results demonstrated that miRNA-181a was downregulated 2.2-fold in HELA/Ara-C cells compared with HELA Cells; B: the enhanced sensitivity to Ara-C was significant higher in crocin plus Ara-C group than that of Ara-C group; C: quantitative RT-PCR results demonstrated that miRNA-181a was upregulated 1.5-fold in HELA/Ara-C Cells plus crocin group and 1.7-fold in HELA/Ara-C Cells plus crocin plus Ara-C group, respectively, compared with HELA/Ara-C cells.

4 Discussion

Currently there are two types of diagnostic tests for cervical cancer screening: Papanikolaou test and HPV test. The first one detects early the precancerous and cancerous cell lesions in order to be effectively treated and the second one infections by HPV types that could lead to cancer. Most of the HPV infections are self-curable and do not cause precancerous cell changes; only chronic infection by specific HPV types could lead to cervical cell abnormalities. If these abnormalities (pre- cancerous or high-grade lesions) are not treated, they may evolve into cervical cancer after many years. (Salzer et al., 2018; Brix et al., 2018; Tanner et al., 2017; Shin et al., 2021). The survival rates have improved remarkably over the past decades, largely due to conventional chemotherapy. However, the side effects of cytotoxic chemotherapy remain significant. Moreover, the drugs resistance is one of the major issues in the management of chemotherapy for patients.

One major mechanism of drug resistance in cancer cells is the defective apoptosis pathway. Recently, more and more findings have established that miRNAs modulate drug resistance of cancer cells, at least in part, through this mechanism. Function research of miRNA-181s was first focused on hematopoietic lineage differentiation in mouse, and mmu-miRNA- 181s was reported to show an obviously high expression at the adult stage, compared with embryonic and early postnatal stages in hematopoietic lineage differentiation (Chen et al., 2004; Sales et al., 2021). Researcher have found that Ara-C- induced drug resistance was associated with downregulation of miR-181a in the cervical cancer cell line HELA. miR-181a might regulate the death of Ara-C-resistant cervical cancer cells by targeting Bcl-2 expression and causing subsequent changes in the apoptosis pathway (Bai et al., 2012; Chatterjee et al., 2015). Recent studies by Fanini et al. showed that miRNA-181a and miRNA-181b might serve as tumor suppressors in human acute monocytic cervical cancer (AML) and human glioma cells, respectively (Bottoni & Calin, 2014; Shi et al., 2008; Iwansyah et al., 2021; Ji et al., 2009). Agents that increase miRNA-181a expression induced apoptosis of AML blasts, while exogenous overexpression of miRNA-181a and miRNA-181b also induced apoptosis of human glioma cells. However, researcher showed that miRNA-181s was highly expressed in EpCAM-positive hepatic cancer stem cells, which was more aggressive, compared with alpha-fetoprotein-positive hepatic cancer cells, and inhibition of miRNA-181 led to a reduction in EpCAM-positive hepatic cancer stem cell quantity and tumor-initiating ability; furthermore, hsa-miRNA-181s was also highly expressed in embryonic livers and in isolated hepatic stem cells. These results related with role miRNA-181s in hepatic stem cells demonstrated that miRNA-181s could be an excellent biomarker for tumor monitor and for the novel targeted drugs exploration, which were consistently with the results in this study.

In this study, we focused on HELA/Ara-C cells and aimed to further investigate the effect and the potential mechanism of crocin on the Ara-C resistance of HELA/Ara-C cells. We found that HELA/Ara-C cells were transfected with the mature miRNA-181s mimic and the control miRNA mimic, respectively. Quantitative RT-PCR results demonstrated that compared with HELA/Ara-C Cells +Ara-C group, miRNA-181a was upregulated 1.5-fold in HELA/Ara-C Cells + crocin group and 1.7-fold in HELA/Ara-C Cells + crocin + Ara-C group, respectively. The other miRNA-181s showed no significant differences. Which were further explaining the potential mechanism of crocin on the drug resistant cell lines.

In summary, this was the first study to show that miRNA-181a may be involved in the development of crocin induced reversing Ara-C resistance in HELA/Ara-C cell lines. Our study may have implications for cancer chemotherapy whose efficiency is often impeded by the development of drug resistance.

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