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ORIGINAL ARTICLE

Antitumor effects of emodin in CACO-2 human colon carcinoma cells are mediated via apoptosis, cell cycle arrest and downregulation of PI3K/AKT signalling pathway

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Summary

Purpose: *Emodin is an important constituent of Rheum emodi, an important medicinal herb. Emodin has been reported to exhibit significant pharmacological potential. Several activities such as anticancer activity have been attributed to emodin. However, the anticancer effects of emodin on colon cancer cells have not been fully studied. Therefore, the present study was designed to investigate the anticancer activity of emodin against the CACO-2 colon carcinoma cells.*

Methods: *The anti-proliferative activity of emodin was assessed by MTT assay. Apoptosis, and cell cycle analysis were carried out by flow cytometry using different fluorescent probes. Expression of proteins was examined by western blotting.*

Results: *The results indicated that emodin reduced the viability of CACO-2 colon cancer cells. The observed IC_{50} for emodin was 30 μM at 24 hrs of incubation. Furthermore, the anticancer effects of emodin were found to be due to induction of apoptosis. Mitochondrial membrane potential (MMP) determination and Bax/Bcl-2 ratio indicated that emodin-induced apoptosis followed the mitochondrial pathway. Emodin could also trigger cell cycle arrest in CACO-2 colon carcinoma cells in a dose-dependent manner. Evaluation of the effect of emodin in PI3/AKT signalling pathway revealed that emodin could inhibit this signalling cascade indicating the potential of emodin as anticancer drug for the treatment of colon cancer.*

Conclusion: *Emodin exhibited potent anticancer effects in CACO-2 human colon carcinoma cells by inducing apoptosis, cell cycle arrest and inhibition of PI3K/AKT signalling pathway.*

Key words: *apoptosis, cell viability, colon carcinoma, PI3/AKT*

Introduction

Cancer is a very complex class of diverse diseases differing in their cause and biology. It involves activation of a number of signalling cascades that initiate cell proliferation, prevent apoptosis and allow the diseased cells to invade different organs of the body [1]. Cancer is one of the four most leading causes of death across the globe and in United States one out of every four deaths is caused by cancer [2]. Colon carcinoma is one of most common types of gastrointestinal cancers and with the change of life style, its incidence has increased drastically. It is believed that colon

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cancer ranks second among the malignant tumor-related deaths [3]. Currently the treatment of colon cancer involves surgery followed by chemotherapy. However, its prognosis is rather poor and the mortality rate is high [3]. Therefore, there is an urgent need to develop novel treatment strategies or explore novel targets for the treatment of this malignancy.

Plants have been used for the treatment of diseases since antiquity [4]. According to reports of WHO, the bulk of Asian and African people are dependent on traditional medicines mainly based on plant-derived molecules or extracts [5]. The ethnopharmacological uses of many of these plants have even been scientifically validated. Among the medicinal plants, *Rheum emodi* belonging to family *Polygonaceae,* is an important source of bioactive molecules, mainly anthraquinones such as emodin and chrysophanol. These constituents of *Rheum emodi* have been reported to exhibit impressive pharmacological properties which include but are not limited to cytotoxic, antimicrobial, antitumor and antidiabetic [6]. Emodin which is an important principal of *Rheum emodi* has been reported to inhibit the TPA-triggered cancer cell invasion [7]. However, the anticancer activity of emodin has not been explored against colon cancer cells. Therefore, the present study was designed to evaluate the anticancer activity of emodin against CaCO-2 human colon cancer cells, along with exploration of its anticancer mechanism by studying its effect on apoptosis induction, cell cycle arrest, MMP and expression of Bcl-2 and Bax.

Methods

Reagents, colon cancer cell line and culture conditions

DAPI, RNase A triton X-100 dimethyl and sulfoxide (DMSO) were procured from Sigma-Aldrich (St.Louis, MO, USA). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Pudong, New District, Shanghai, China). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine and antibiotics were obtained from Invitrogen Life Technologies (California, USA). Human colon cancer cell line CACO-2 was obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and maintained in a humidified atmosphere containing 5% CO_2.

MTT cell viability assay

The antiproliferative effects of emodin were evaluated against CACO-2 cells at concentrations ranging from 0-200 µM. Emodin was dissolved in DMSO. Briefly, cells at a density of 10^4 cells/well were cultured in 96-well plates for 24 hrs. After incubation 20 µL of MTT (2.5 mg/ml) were added and incubated for another 4 hrs. Finally the absorbance was determined at 570 nm using Eliza Plate Reader.

Apoptosis assay

Colon cancer CACO-2 cells were seeded at a density of 2×10^5 cells/well in 96-well plates, treated with varying concentrations of emodin and incubated for 24 hrs. DAPI staining was carried by incubating the cells with DAPI. The cells were then washed with PBS, fixed in 10% formaldehyde and washed again with PBS. The DAPI-stained cells were then examined by fluorescence microscope. For annexin V/propidium iodide (PI) a similar procedure as that of DAPI was followed except for the cells stained with annexin V/PI and investigated by flow cytometry.

Mitochondrial membrane potential determination

CACO-2 were cultured at a density of 2×10^5 cells/well in a 6-well plate, incubated for 24 hrs and treated with 0, 15, 30 and 60 µM emodin for 24 hrs at 37°C. Afterwards, the cells were collected, washed twice by PBS and re-suspended in 500 µl DiOC6 (1 µmol/l) for estimation of $\Delta\Psi m$ at 37°C in the dark for 30 min. The samples were then analyzed instantly using flow cytometry.

Analysis of cell cycle distribution by propidium iodide staining

Following 24-h incubation, exponentially growing colon carcinoma cells (2×10^5 cells/ml/well) were treated with 0, 15, 30 and 60 µM concentrations of emodin for 24 hrs. The cells were then collected by trypsin treatment followed by washing in cold phosphate buffer saline (PBS). The cells were then fixed with 70% ethanol and then washed again with cold PBS. The cells were then treated with 40 µg of RNase for 1.5 hrs at 37°C and stained afterwards with 5 µg of PI in the dark for 20 min and finally examined by FACS Aria II flow cytometer (BD Biosciences, San Jose, USA).

Western blotting

CACO-2 colon carcinoma cells were lysed in RIPA buffer and protien extracts were collected. Equal protein extracts from each group were run on SDS PAGE and then transferred to a polyvinylidene fluoride membrane. This was followed by blocking with 5% non-fat milk and incubation at 25°C for 1 hr. Thereafter, the membranes were incubated with a specific primary antibody at 4°C overnight. This was followed by washing in washing buffer and incubation for 1 hr with the suitable secondary antibody. The protein bands of interest were visualised by ECL Advanced Western Blot Detection Kit (Amersham Place, Buckinghamshire, UK).

Statistics

Experiments were carried out in triplicate and presented as mean ± SD. GraphPad 7 was used for statistical analyses and the values were considered significant at $p<0.05$.

Results

Emodin exerted antiproliferative activity on colon carcinoma cells

In the present study the antiproliferative activity of emodin (Figure 1) at varying concentrations (0-200 μM) was evaluated by MTT assay. The results of this assay showed a concentration-dependent inhibition of viability of CACO-2 colon carcinoma cells (Figure 2). Evaluation of the IC_{50} value of emodin at 24 hrs of incubation showed a value of 30 μM against CACO-2 cells.

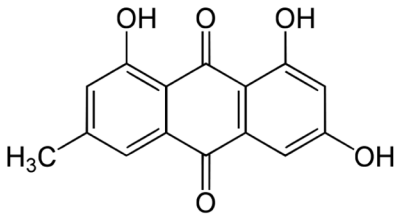

Figure 1. Chemical structure of emodin.

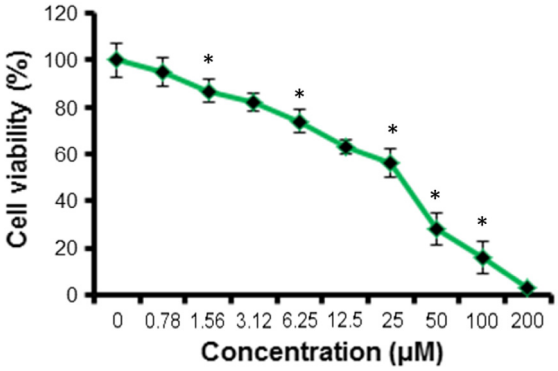

Figure 2. Assessment of cell viability by MMT assay. Colon cancer cell viability decreased with increasing doses of emodin. The results are mean ±SD of three biological experiments. Values were considered significant at *p<0.05.

Emodin triggered apoptosis in colon carcinoma cells

Since the results of MTT assay revealed significant anticancer effects of emodin we investigated whether emodin exerts antiproliferative effects via induction of apoptosis. Consistent with this the results of DAPI showed marked signs of apoptosis induced by emodin (Figure 3). Furthermore, to estimate the apoptotic cell population we carried out annexin V/PI staining. The results revealed that the apoptotic cell populations increased significantly from 1.85% in the control to 42.66% at 60 μM concentration (Figure 4).

Emodin-induced apoptosis followed the mitochondrial pathway

To investigate whether emodin-triggered apoptosis followed the mitochondrial pathway, we determined MMP. The results showed that emodin decreased the MMP in a concentration-dependent manner (Figure 5). Moreover, examination of Bax and Bcl-2 expression revealed that emodin could significantly upregulate the expression of Bax which was associated with concomitant downregulation of Bcl-2 (Figure 6).

Emodin induced cell cycle arrest

Cell cycle arrest is considered as an important mechanism by which anticancer agents exert their antiproliferative effects. Therefore, we investigated

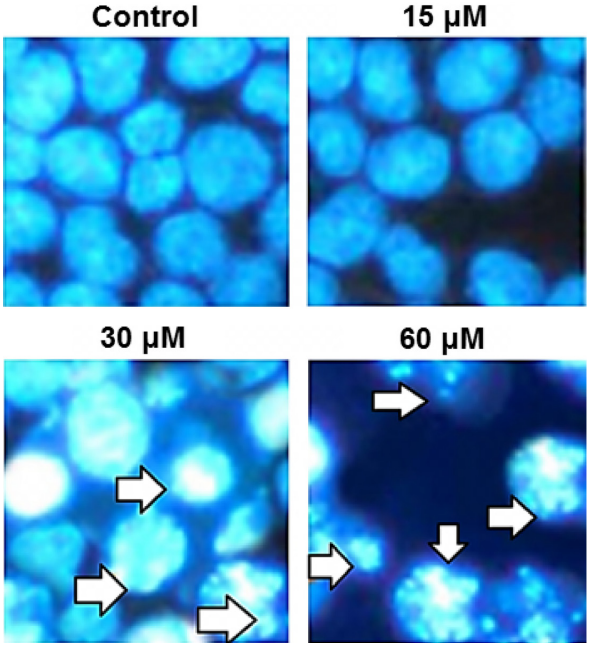

Figure 3. DAPI staining reveals induction of apoptosis at the indicated doses. Experiments were carried out in triplicate. Arrows indicate apoptotic cells appearing with bright fluorescence.

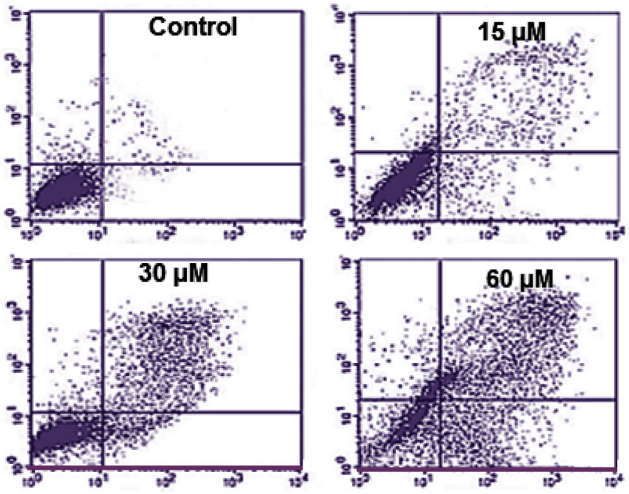

Figure 4. Estimation of apoptotic cell populations by annexin V/PI staining. Experiments were carried out in triplicate. The Figure shows the apoptotic cells (both early and late apoptosis) and reveals a significant increase of these cells with increasing emodin dose.

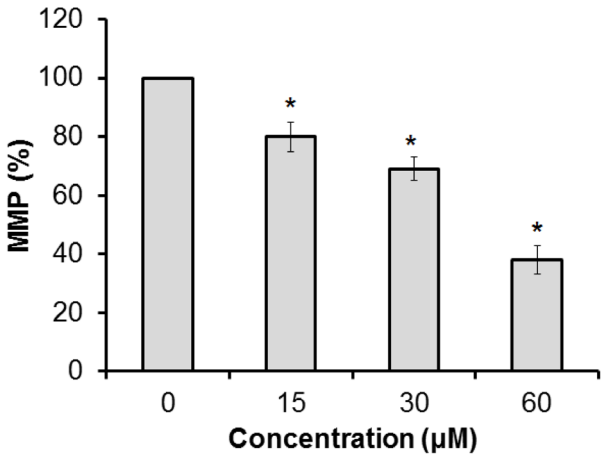

Figure 5. Estimation of MMP at indicated doses. The results are mean ±SD of three biological experiments. Values were considered significant at *p<0.05. There is a considerable decrease of MMP with increasing doses of emodin.

the effects of emodin on cell cycle distribution of CACO-2 cells (Figure 7). The results showed that emodin treatment caused significant increase in the G2 phase, indicative of G2/M cell cycle arrest.

Emodin inhibited PI3/AKT signaling pathway

PI3/AKT signalling pathway is an important cascade that is upregulated in cancer cells. It has been shown to be involved in the progression and tumorigenisis of several cancers. Out results indicated that emodin could downregulate the expression of p-PI3 and p-AKT proteins (Figure 8) indicating the potential of emodin as anticancer agent.

Discussion

Plants have been used as source of medicines for the treatment of diseases and disorders since times immemorial [8]. Even today a number of FDA approved drugs has been isolated from plants. Anticancer drugs such as taxanes, vinca alkaloids (vinblastine, vincristin) have plants as their source [9]. *Rheum emodi* is an important medicinal plant that has been widely used in different traditional systems of medicine [4]. Emodin is an important active constituent of *Rheum emodi* and in the present study we examined the anticancer activity of emodin against CACO-2 colon cancer cells. Our results indicated that emodin could significantly inhibit the proliferation of colon cancer cells and exhibited an IC_{50} of 30 μM. The antiproliferative effects of emodin were found to be concentration-dependent. Our results are supported by the results obtained in other studies wherein emodin has been reported to exhibit anticancer activity against a range of cancer cells [10]. After further investigation, we observed that the emodin

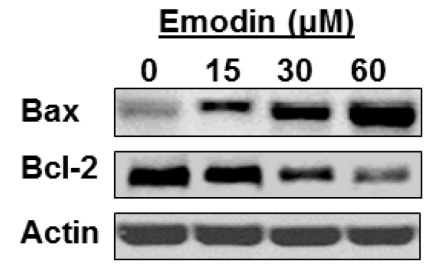

Figure 6. Effect of emodin on Bax and Bcl-2 expression determined by western blotting. Experiments were carried out in triplicate. Emodin could significantly upregulate the expression of Bax which was associated with concomitant downregulation of Bcl-2.

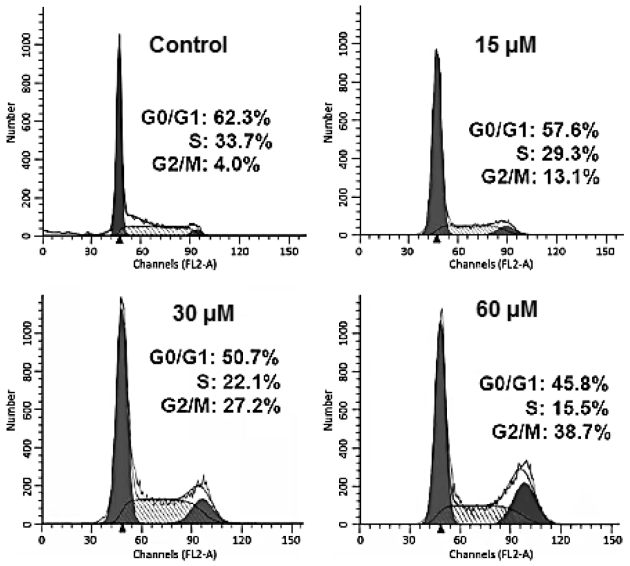

Figure 7. Effect of emodin at indicated doses on cell cycle phase distribution as determined by flow cytometry. Experiments were carried out in triplicate. The results showed that emodin caused significant increase of cells in the G2 phase, indicative of G2/M cell cycle arrest.

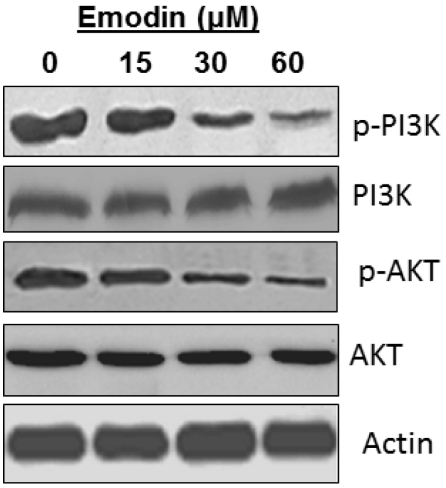

Figure 8. Effect of emodin on PI3K/AK protein expression determined by western blotting. Experiments were carried out in triplicate. Emodin downregulated the expression of p-PI3K and p-AKT proteins, indicating the potential of emodin as anticancer agent.

induced apoptosis in CACO-2 colon cancer cells in a concentration-dependent manner, as evidenced from the results of DAPI staining. The results of annexin V/PI staining indicated that emodin increased the apoptotic cell populations from 1.85% in the control to 42.66% at 60 μM concentration. Our results are well supported by a previous study wherein emodin has been reported to trigger apoptosis in HK-2 cells [11].

To further confirm whether the emodin-induced apoptosis followed the mitochondrial pathway, we determined the MMP of emodin-treated cancer cells. The results showed significant reduction in the MMP which was also associated with upregulation of Bax and downregulation of Bcl-2 expression. These results indicate that emodin-induced apoptosis followed the mitochondrial apoptotic pathway.

Cell cycle arrest is a powerful mechanism for suppression of the proliferation of cancer cells. The anticancer activity of several of the anticancer drugs has been attributed to the induction of cell cycle arrest [12]. We therefore examined the effect of emodin on the cell cycle phase distribution of human colon carcinoma CACO-2 cells. The results indicated that emodin could induce G2/M cell cycle arrest of CACO-2 cells as evidenced from the increased number of cells in G2/M phase. Previous studies have also shown that emodin and its components trigger cell cycle arrest in a panel of cell lines [13].

The important PI3/AKT signalling pathway has been reported to be upregulated in several cancer types. Furthermore, this pathway has been shown to be involved in the progression and tumorigenesis of cancer cells [15]. In the present study we observed that emodin could suppress the expression of some of the key proteins indicating the potential of emodin as anticancer agent.

Conclusion

We conclude that emodin exhibits significant inhibitory activity on the growth of CACO-2 colon cancer cells. Its anticancer effects were mainly due to induction of apoptosis and cell cycle arrest. Moreover, it could also suppress the PI3/AKT signalling cascade of CACO-2 cells, suggesting the potential use of emodin in the treatment of colon carcinoma.

Conflict of interests

The authors declare no conflict of interests.

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