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ORIGINAL ARTICLE

Apigenin inhibits *in vitro* and in *vivo* tumorigenesis in cisplatin-resistant colon cancer cells by inducing autophagy, programmed cell death and targeting m-TOR/PI3K/Akt signalling pathway

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Summary

Purpose: *Colon cancer is one of the deadly malignancies and the second most frequent cancer in the world. The development of drug resistance and dearth of the viable drug options forms an obstacle in the treatment of colon cancer. Herein, the anticancer potential of Apigenin was examined against cisplatin-resistant colon cancer cells.*

Methods: *The proliferation rate of the cisplatin-resistant colon cancer cell line HT-29 was assessed by WST-1 assay. Autophagy was detected by electron microscopy. Apoptotic cell death was analysed by propidium iodide (PI) staining. Cell cycle analysis was performed by flow cytometry. Protein expression was determined by immuno blotting. Xenografted mice models were used for in vivo evaluation of Apigenin.*

Results: *The results showed that Apigenin could considerably inhibit the proliferation of colon cancer cells. The anticancer activity of Apigenin against the HT-29 colon cancer cells was found to be due to induction of autophagy and apoptosis. The Apigenin-triggered apoptosis and autophagy were also linked with alteration in the apoptosis and autophagy-related protein expression. Furthermore, it was found that Apigenin could inhibit the m-TOR/PI3K/AKT signalling pathway in the cisplatin-resistant colon cancer cells. The effects of Apigenin were also examined in vivo in xenografted mice models and it was revealed that Apigenin inhibited the growth of xenografted tumors.*

Conclusions: *Taken together, these results indicate that Apigenin could inhibit the growth of cisplatin-resistant colon cancer cells in vitro and in vivo and may be used for the improvement of therapy of colon cancer.*

Key words: *autophagy, apoptosis, cell cycle, apigenin, colon cancer*

Introduction

Colon cancer causes tremendous mortality world over. The incidence of colon cancer has increased at an alarming rate and is now the second most widespread type of cancer in the world and fifth in USA [1,2]. The different adverse effects of the chemotherapeutic agents and minimal choice of the effective drugs create limits in the treatment of colon cancer [3]. Recent studies have shown that molecules derived from edible plants can be used as safer anticancer drugs [4]. Plants form a ubiquitous source of flavonoids and it is believed that diets that contain high amounts of flavonoids reduce

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the risk of cancer development [5]. Pharmacological studies have shown that flavonoids possess a number of bioactivities, including anticancer activity [6]. Several of the flavonoids have been reported to halt the proliferation of cancer cells and thus may be considered essential for the development of novel therapies for colon cancer [7]. Apigenin is an important flavonoid that has been shown to inhibit the growth of several types of cancer cells [8,9]. However, the anticancer effects of Apigenin against the cisplatin-resistant colon cancer cells have not been investigated and the underlying mechanism is still not known. It was found that Apigenin could suppress the growth of cisplatin-resistant colon cancer. The anticancer effects of Apigenin were found to be related to induction of autophagy and programmed cell death (apoptosis). The mTOR/PI3K/AKT is one of the important signalling pathways [10,11] and has been shown to have a role in the development, progression and tumorigenesis in many types of cancers and as such it is considered as an important therapeutic target [12,13]. It was observed that Apigenin blocked this pathway [12,13]. The main purpose of this study was to investigate the *in vitro* and *in vivo* anticancer effects of Apigenin in cisplatin-resistant colon cancer cells along with the study of its effects on autophagy, apoptosis and mTOR/PI3K/Akt signalling pathway.

Methods

Cell lines and culturing conditions

The cisplatin-resistant colon cancer cell line HT-295 was procured from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium in CO_2 incubator (Thermo Scientific) at 37°C with 98% humidity and 5% CO_2.

WST-1 assay of cell proliferation

Apigenin's anticancer activity was assessed in cisplatin-resistant colon HT-29 cell line by WST-1 assay. In brief, the colon cancer cells were cultured at a density of 2.5×10^5 cells/well in 96-well plates and subjected to treatment with varied concentrations of Apigenin. This was followed by incubation of the colon cancer cells with WST-1 for 3 h at 37°C and the proliferation rate was determined by assessing the absorbance at 450 nm. Cell morphology was also examined by phase-contrast microscopy as described previously [14].

Electron microscopy

The effects of the natural flavonoid Apigenin on the cisplatin-resistant colon cancer cells were examined by electron microscopy. Firstly, the colon cancer cells were administered 0, 20, 40 and 80 µM Apigenin for 24 h. The cells were then collected by trypsinization and washed with DMEM medium, which was followed by fixation in glutaraldehyde (2%) in phosphate buffer (0.1 M). The cells were then post-fixed in osmium tetroxide (1%). This was followed by treatment of the cells with ethanol and embedding in resin. Thin sections were then cut with the help of an ultramicrotome and subjected to electron microscopy.

Propidium iodide staining for the detection of apoptosis

PI staining was employed for the detection of apoptosis. Firstly, the cisplatin-resistant colon cancer cells (0.6×10^6) were grown in 6-well plates. Following an incubation period of around 12 h, the cisplatin-resistant colon cancer HT-29 cells were subjected to Apigenin treatment for 24 h at 37°C. The cell cultures were then centrifuged and the pellets were washed with phosphate buffered saline (PBS). Next, the cells were stained with PI, centrifuged and PBS-washed. Finally, the nuclear morphology of the stained cells was examined by confocal microscopy.

Western blotting

To determine the expression of the selected proteins in the Apigenin-treated cisplatin-resistant colon cancer cells, the cells were subjected lysis with RIPA buffer and the protein content of each lysate was estimated by bicinchoninic acid (BCA) assay. The samples were then loaded on SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for 24 h. Following this, the membranes were incubated with HRP-conjugated secondary antibody for 50 min at 25°C. Enhanced chemiluminescence reagent was used to visualise the protein bands.

In vivo study

Apigenin was also evaluated for its anticancer *in vivo* in xenografted mice. In brief, 4-week-old mice (nude, BALB/c) were maintained in the animal house as per the NIH guidelines. The mice were then injected with 5×10^6 HT-29 cells subcutaneously at the left flank. The mice in each group (n=5) were injected intraperitoneally with DMSO (0.1%) dissolved Apigenin and diluted with 100 µL normal saline at 35 mg/kg and this was taken as the first day of the experiment. Apigenin was given to the xenografted mice thrice a week while the control mice were given DMSO (0.1%) in normal saline only. At the end of 6 weeks, the mice were euthanized, and the tumors were removed and used for analysis.

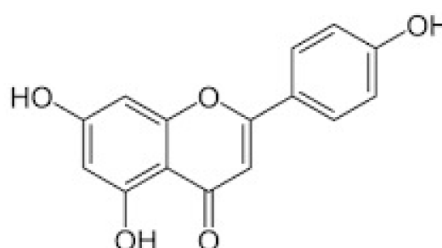

Figure 1. Chemical structure of Apigenin.

Statistics

Data are shown as mean±SD. Statistical analyses were done using Student's t-test with GraphPad prism 7 software. P values<0.05 were considered as statistically significant

Results

Growth inhibitory effects of Apigenin in colon cancer cells

To confirm the antiproliferative effects of Apigenin, the WST-1 assay was performed (Figure 1). Apigenin showed significant antiproliferative effects on the cisplatin-resistant colon cancer cells which were found to be dose-dependent (Figure 2). The IC_{50} of Apigenin against the HT-29 cisplatin-resistant colon cancer cells was 30 µM. In addition, it was found that the anticancer effects of Apigenin on the colon cancer cells were concentration-dependent. Furthermore, it was observed that Apigenin caused nuclear condensation, membrane shrinkage and blebbing of the HT-29 cells (Figure 3).

Induction of autophagy and programmed cell death in colon cancer cells by Apigenin

Electron microscopic analysis of Apigenin-treated cisplatin-resistant colon HT-29 cells revealed that Apigenin treatment prompted the development of autophagosomes in the colon cancer cells, indicating that Apigenin induces autophagy (Figure 4). In addition, Apigenin also caused shrinkage of the nuclei of HT-29 cells, suggestive of apoptosis. To confirm autophagy, the expression of autophagy-related proteins was investigated and it was revealed that Apigenin caused increase of Beclin-1 and LC3-II and suppression of p62 expression. However, no effects were found on the expression of LC3-I and Vps34 (Figure 5). The fact that Apigenin also induces apoptosis was validated by PI staining which showed apparent changes in the nuclear morphology of the HT-29 cells (Figure 6). The increased expression of Bax and decreased expression of the Bcl-2 in HT-29 cells further confirmed the activation of programmed cell death (Figure 7).

Inhibition of mTOR/PI3K/AKT signalling pathway by Apigenin

Apigenin-treated cells were also used for investigation of the effect of Apigenin on the mTOR/PI3K/AKT signalling pathway. It was found that Apigenin treatment resulted in dose-dependent reduction in the expression of p-mTOR, p-PI3K and p-AKT, while no apparent effect was seen on the expression of mTOR, PI3K and AKT (Figure 8).

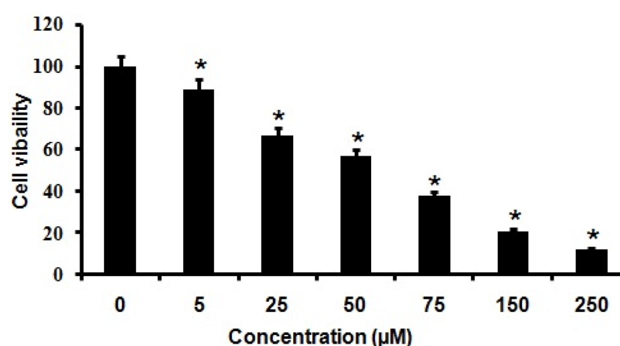

Figure 2. Effect of Apigenin on the viability of the HT-29 cells as determined by WST-1 assay. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

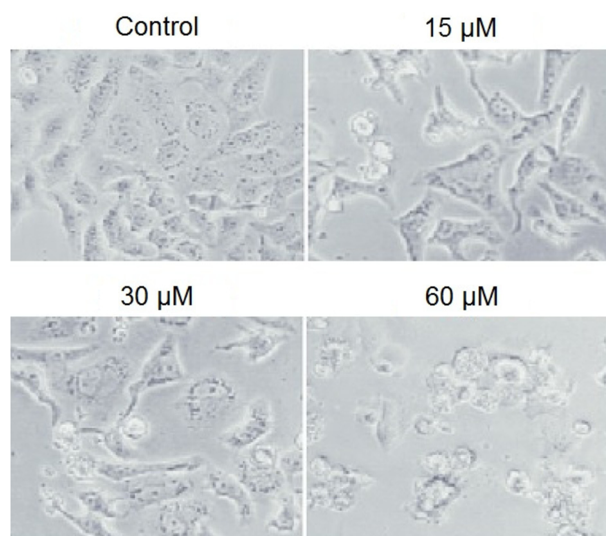

Figure 3. Effect of Apigenin on the morphology of the colon cancer cells. The Figure shows that Apigenin caused nuclear condensation, membrane shiknage and blebbing. The experiments were performed in triplicate.

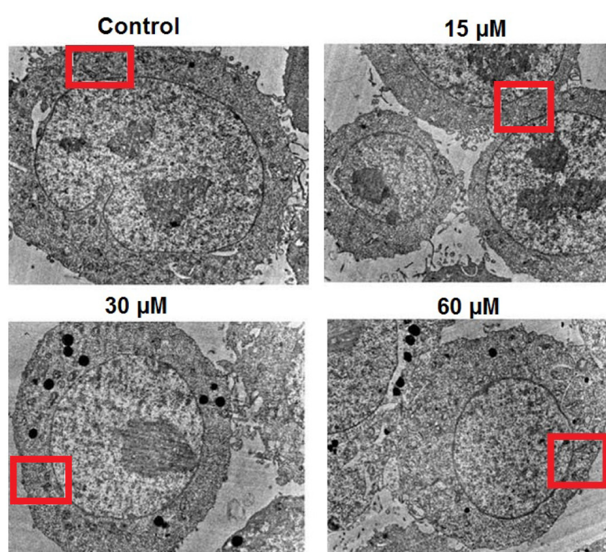

Figure 4. Electron microscopy images of Apigenin-treated HT-29 cells showing induction of autophagy in a dose-dependent manner. The experiments were performed in triplicate.

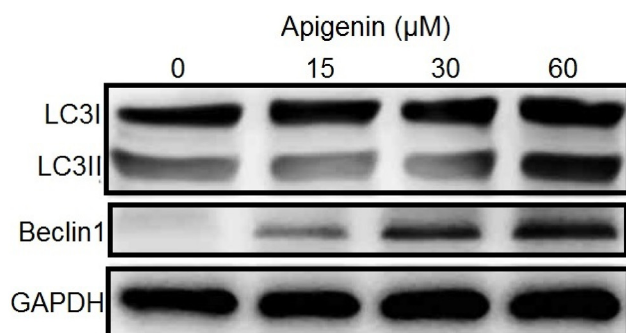

Figure 5. Effect of Apigenin on the autophagy-related protein expression as revealed by western blot analysis. Apigenin caused increase of Beclin 1 and LC3-II and suppression of p62 expression. Notably, no effects were found on the expression of LC3-I. The experiments were performed in triplicate.

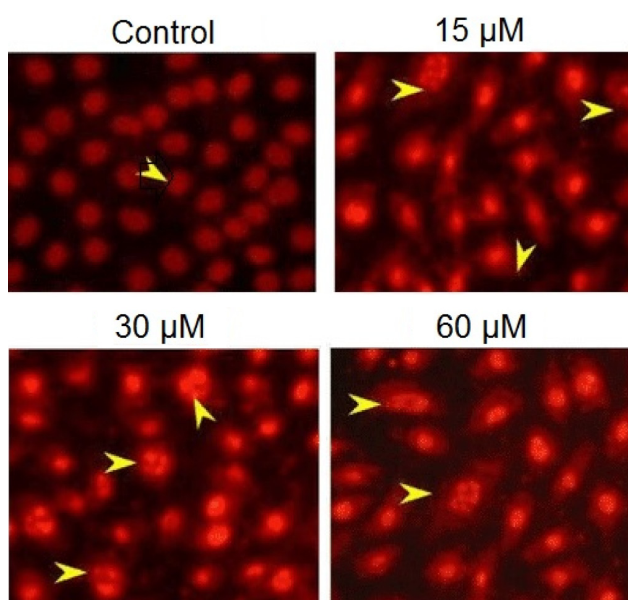

Figure 6. Propidium iodide staining images showing induction of apoptosis by Apigenin on the HT-29 images. Apigenin caused apparent changes in the nuclear morphology of the HT-29 cells. Arrows indicate apoptotic cells. The experiments were performed in triplicate.

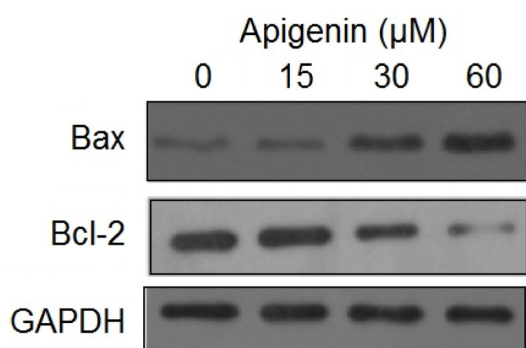

Figure 7. Effect of Apigenin on the expression of Bax and Bcl-2 proteins as depicted by western blot analysis. Apigenin led to increased expression of Bax and decreased expression of Bcl-2 in HT-29 cells. The experiments were performed in triplicate.

Hence, the results suggest that Apigenin blocks the mTOR/PI3K/AKT signalling pathway in HT-29 cells.

Apigenin inhibits tumor growth in vivo

The effects of Apigenin were also examined *in vivo* in xenografted mice models and the results indicated that Apigenin at the dosage of 35 mg/kg considerably suppressed the growth of the xenografted tumors. In addition, Apigenin inhibited the tumor weight and volume concentration-dependently (Figure 9A and B).

Discussion

Colon cancer is the second most prevalent type of malignancy in the world [15]. This cancer represents a problematic area due to inefficient treatment options and diagnosis at advanced stages [16]. In addition, the anticancer drugs that are being used for treating colon cancer are a source of important side effects which negatively impact the patient quality of life [17]. Molecules derived from plants have attained extraordinary attention in the recent past due to their comparatively lower toxic effects. Hence, the focus on the researchers across the globe is to identify and screen natural products against cancer cells for developing efficient drugs to target colon and other cancers [18]. In the present study, the antiproliferative effects of Apigenin were determined in cisplatin-resistant colon cancer

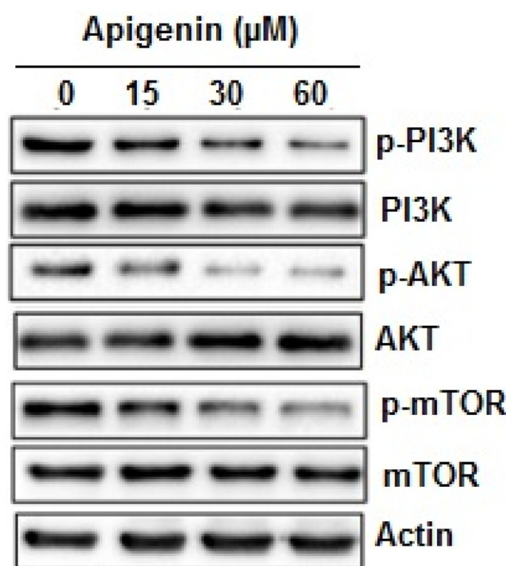

Figure 8. Effect of Apigenin on the mTOR/PI3K/AKT signalling pathway as depicted by western blot analysis. Apigenin treatment resulted in a dose-dependent reduction of the expression of p-mTOR, p-PI3K and p-AKT, whereas no apparent effect was seen on the expression of mTOR, PI3K and AKT. The experiments were performed in triplicate.

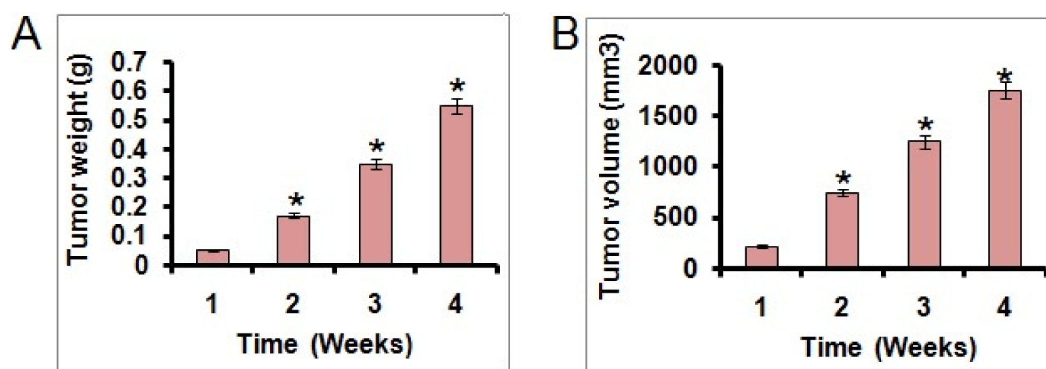

Figure 9. Effect of Apigenin on tumor growth of xenografted tumors. **A:** Tumor volume and **B:** Tumor weight. Apigenin inhibited the tumor weight and volume concentration-dependently. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

cells. It was found that Apigenin treatment of HT-27 cells resulted in decline of proliferation rate. In the studies carried out previously, flavonoids have also been reported to inhibit the growth of cancer cells by triggering apoptosis and autophagy [19]. Autophagy and apoptosis are two processes that maintain the tissues' homeostasis and help in the elimination of unwanted, harmful or cancer cells [20]. Herein, it was found that Apigenin activated both autophagy and apoptotic cell death of the HT-29 colon cancer cells. This was also associated with changes in the expression of autophagy as well as in the apoptosis-related protein expression. Previous studies have indicated that several of the anticancer molecules induce autophagy as well as apoptosis of cancer cells [20]. In cancer tissues/cells several of the signalling pathways are aberrantly activated and mTOR/PI3K/AKT is one such pathway which has been shown to play essential role in the proliferation of cancer cells [21]. In this study we found that Apigenin could inhibit the expression of p-mTOR, p-PI3K, and p-AKT in HT-29 cells concentration-dependently. Finally, it is imperative to evaluate the anticancer potential of such molecules *in vivo* in order to consider them as potential lead molecules. In the xenografted mice models it was found that Apigenin inhibits the growth of xenografted tumors, suggestive of its potent anticancer potential.

Conclusions

In conclusion, Apigenin exerts growth inhibitory effects on the cisplatin-resistant colon cancer cells by autophagy and apoptotic cell death. In addition, it also suppressed the tumor growth *in vivo*. Hence, Apigenin may prove to be a vital therapeutic agent and needs further in-depth investigation.

Conflict of interests

The authors declare no conflict of interests.

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