

ORIGINAL ARTICLE

A flavone, Wogonin from *Scutellaria baicalensis* inhibits the proliferation of human colorectal cancer cells by inducing of autophagy, apoptosis and G2/M cell cycle arrest via modulating the PI3K/AKT and STAT3 signalling pathways

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Summary

Purpose: The purpose of this study was to examine the anticancer effects of a flavone from *Scutellaria baicalensis* wogonin against a panel of colorectal cancer cells.

Methods: The SW1417, SW48, DLD-1, HCT-15, LS-180 and CCD-18Co cell lines were used for the evaluation of the anticancer effects of wogonin. WST-1 and colony formation assays were used for cell viability assessment. Cell cycle analysis was assessed by flow cytometry. Autophagy was detected by electron microscopy. Apoptosis was detected by acridine orange (AO)/ethidium bromide (EB) staining. Cell protein expression was checked by western blotting.

Results: The cytotoxic effects of wogonin were comparatively negligible against the normal CCD-18Co cells with an IC_{50} of $>100 \mu M$. Investigation of the mechanism of action

revealed that wogonin exerts growth inhibitory effects on the SW48 colorectal cancer cells by autophagic and apoptotic cell death. This was also accompanied with upregulation of autophagic proteins such as LC3II and Beclin 1 as well as the apoptotic proteins such as caspase 3, 8 and 9 and Bax expressions. Wogonin also induced arrest of the SW48 cells at the G2/M check point of the cell cycle. In addition, wogonin could also inhibit the PI3K/AKT and STAT3 signal transduction pathways.

Conclusion: These results suggest that wogonin exerts potent anticancer effects on colorectal cancer cells and may prove essential in the management of colorectal cancer.

Key words: wogonin, colorectal cancer, autophagy, apoptosis

Introduction

Colorectal cancer being the fourth leading cause of cancer-related mortality is one of the prevalent types of cancers. It is also ranked as third common type of cancer and around 1.4 million new cases of colorectal cancer are reported every year [1]. In 2013, around 0.7 million deaths were reported to be due to colorectal cancer through the world [2]. The incidence of colorectal cancer is believed that will increase by 60% till 2030 [3]. Late diagnosis and lack of potent and safe chemo-

therapeutic drugs built an obstacle in the treatment of colorectal cancer [4]. Nature has bestowed mankind with an inexhaustible array of chemical scaffolds. These amazing chemical entities stand as an exceptional source of drugs for the management of human diseases [5]. Although the use of herbal originates from ancient times, use of pure isolated compounds started only in the 19th century [6]. Since, then a wide array of molecules have been isolated, evaluated and used for the treatment

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Received: 05/09/2018; Accepted: 02/11/2018

of several diseases and disorders [7]. Among plant metabolites, the ubiquitous flavonoids have shown promising potential for drug development. Flavonoids are common components of human diet and it is believed that consumption diet rich in flavonoids lowers the risk of cancer development [8]. Wogonin is an important flavone that is believed to possess strong pharmacological potential [9]. It is prevalently isolated from the medicinal herb *Scutellaria baicalensis* and has been reported to halt the growth of several types of cancer cells [10]. For example, wogonin has been shown to inhibit the growth of breast cancer cells by the 5-LO/BLT2 signalling [11]. However, the anticancer effects of wogonin are yet largely unknown. Herein, we report the anticancer effects of wogonin against a panel of colorectal cancer cell lines and one normal cell line.

Methods

WST-1 Cell viability and colony formation assay

The anticancer effect of wogonin was assessed on colorectal (SW48, DLD-1, HCT-15, LS-180) and normal (CCD-18Co) cell lines by WST-1 assay. In brief, the SW48 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 wogonin. This was followed by incubation of the SW48 cells with WST-1 for 3 h at 37°C and the proliferation rate was determined by taking the absorbance at 450 nm. Cell morphology of the wogonin-

treated SW48 cells was also examined by phase-contrast microscopy. The effect of wogonin on the formation of SW48 colonies was investigated as described earlier [12].

Electron microscopy

The induction of autophagy in wogonin-treated colorectal cancer cells was assessed by electron microscopy. In brief, the colorectal SW48 cancer cells were treated with 0, 4, 8 and 16 μ M wogonin for 24 h. The cells were collected by trypsinization and washed with phosphate buffered saline (PBS) 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 using an ultramicrotome and subjected to electron microscopy.

Table 1. Anticancer effects of wogonin on different colorectal cancer cell lines as depicted by WST-1 assay and expressed as IC_{50}

| S. No. | Cell line | IC_{50} (μ M) |
|--------|-----------|----------------------|
| 1 | SW1417 | 16 |
| 2 | SW48 | 8 |
| 3 | DLD-1 | 16 |
| 4 | HCT-15 | 32 |
| 5 | LS-180 | 32 |
| 6 | CCD-18Co | >100 |

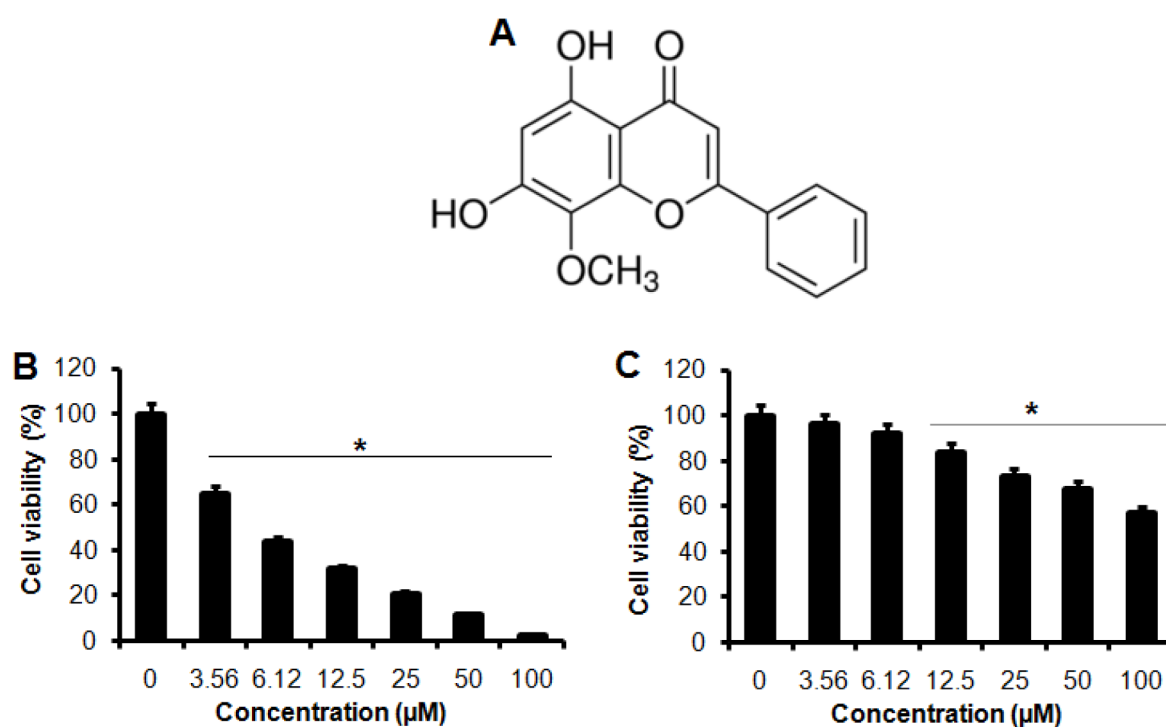


Figure 1. A: Chemical structure of wogonin. B: Effects of wogonin on the viability of the SW48 colorectal cancer cells and C: normal CCD-18Co colorectal cells. The values represent mean \pm SD of three experiments (* $p < 0.05$).

Acridine orange and ethidium bromide (AO/EB) staining for apoptosis

For AO/EB staining, the colorectal cancer SW48 cells (0.6×10^6) were grown in 6-well plates. Following a 12-h incubation, the SW48 cells were subjected to wogonin treatment for 24 h at 37°C. As the cells sloughed off, 25 μ l of cell culture were put onto glass slides and stained

with AO and EB solution (1 μ l). The slides were cover-slipped and examined with a fluorescent microscope.

Cell cycle analysis

After incubating the colorectal SW48 cells with varied concentrations of wogonin (0, 4, 8 and 16 μ M) for 24 h the cells were washed with phosphate buffered saline (PBS). Afterwards, the SW48 cells were stained with propidium iodide (PI) and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Western blotting

The SW48 cells were firstly washed with ice-cold PBS and suspended in a lysis buffer at 4°C and then at 95°C. Afterwards, the protein content of each cell extract was checked by Bradford assay. About 40 μ g of protein were loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membrane was then subjected to treatment with tris-buffered saline (TBS) and then exposed to primary antibody at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Results

Wogonin selectively inhibits the growth of colorectal cancer cells

The growth inhibitory effects of wogonin (Figure 1A) were assessed by WST-1 assay on a panel colorectal cancer cell lines and a normal cell line, as described in the Methods section. It was found that wogonin triggers anti-proliferative effects on all the colorectal cancer cell lines (Table 1). The maximum anti-proliferative effects were observed against the SW48 cells with an IC_{50} of 8 μ M (Figure 1B). Nonetheless, the IC_{50} of wogonin was found to be comparatively higher against the normal CCD-18Co colorectal cells ($IC_{50} > 100$ μ M) (Figure 1B). In addition, it was found that the anticancer effects of wogonin on the colorectal cancer cells were concentration-dependent and wogonin also led to morphological changes in the SW48 cells (Figure 2). Additionally, wogonin exerted suppressive effects on the colony formation of SW48 cells concentration-dependently (Figure 3).

Wogonin induces both autophagy and apoptosis in colorectal cancer cells

To ascertain if wogonin prompts autophagic cell death of the SW48 colorectal cancer cells, the wogonin-treated SW48 cells were examined by electron microscopy. It was found that wogonin triggered the formation of autophagosomes in these cells, indicative of autophagy (Figure 4). In addition, wogonin also caused shrinkage of the

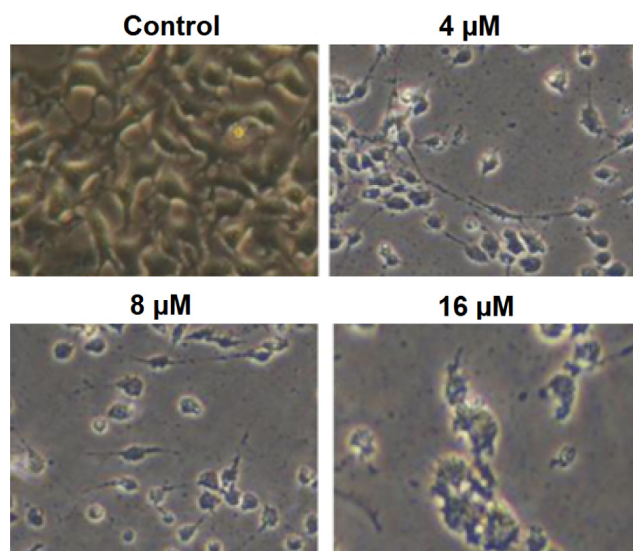


Figure 2. Effects of wogonin on the morphology of the SW48 colorectal cancer cells. The Figure shows that wogonin causes morphological changes of SW48 cells in a concentration-dependent manner. The experiments were performed in triplicate.

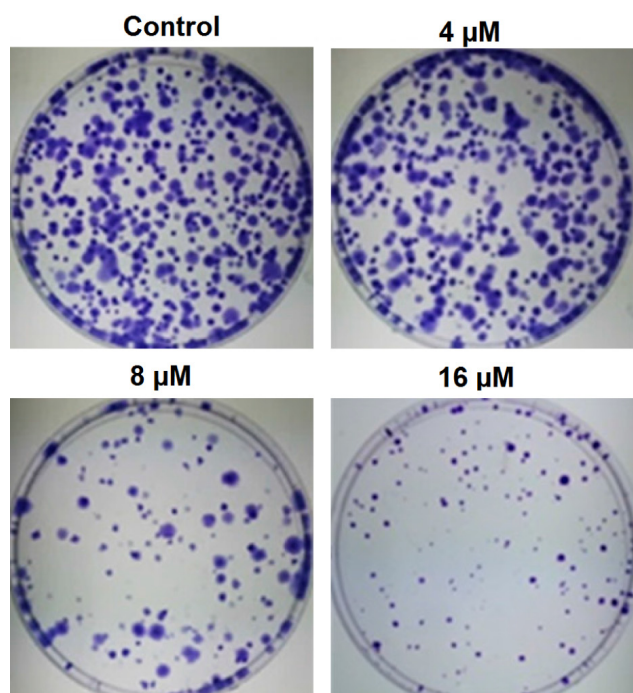


Figure 3. Effects of wogonin on the colony formation of the SW48 colorectal cancer cells. The Figure shows that wogonin inhibits the colony formation of SW48 cells concentration-dependently. The experiments were performed in triplicate.

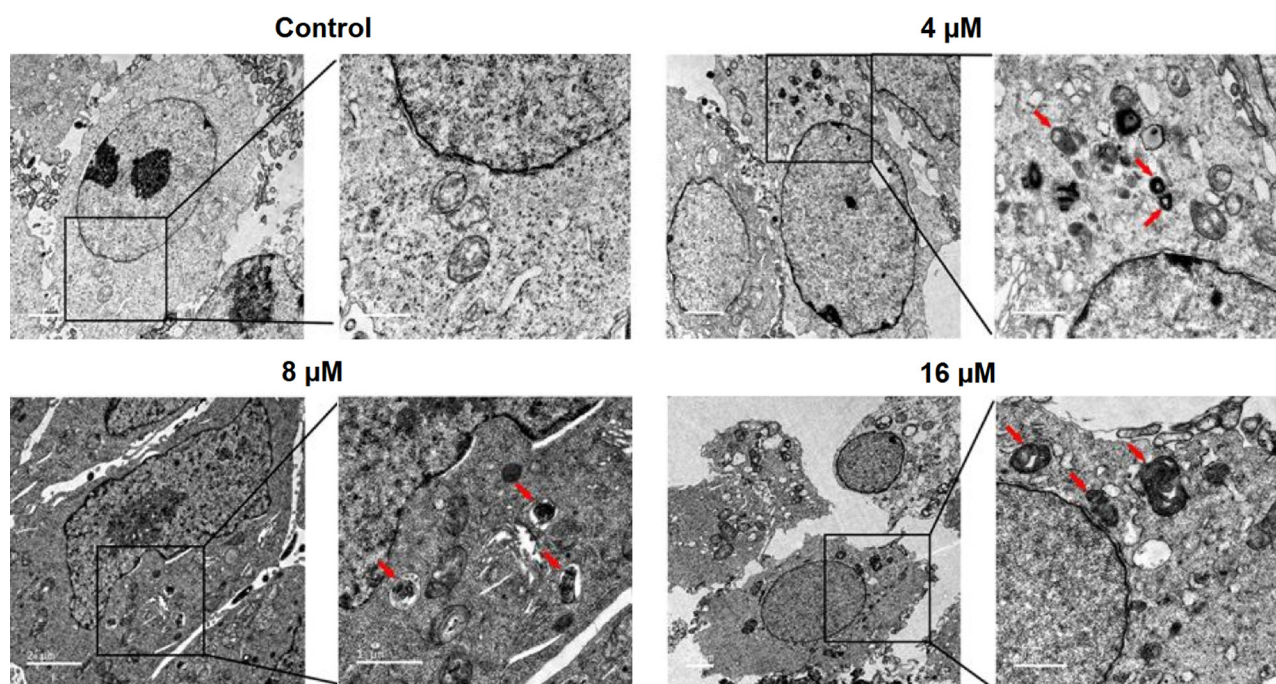


Figure 4. Electron micrographs showing that wogonin induces autophagy in SW48 colorectal cancer cells in a concentration-dependent manner (arrows show autophagosomes). The experiments were performed in triplicate.

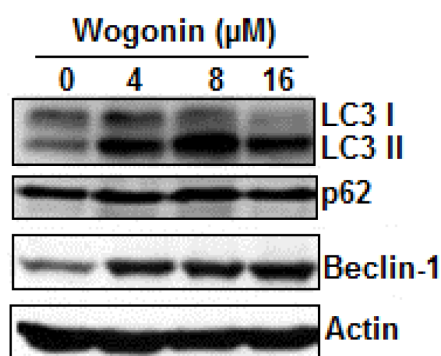


Figure 5. Effect of wogonin on autophagy-related protein expression in SW48 colorectal cancer cells as depicted by western blotting. The Figure shows that wogonin enhances the expression of LC3BII and p62 in a concentration-dependent manner. The experiments were performed in triplicate.

nuclei of the SW48 cells, again indicative of apoptosis. To confirm wogonin-induced autophagy the expression of autophagy associated proteins was examined and it was revealed that wogonin caused increase of Beclin-1 and LC3-II expression. Nonetheless, no effects were found on LC3-I and p62 expression (Figure 5).

Next, the wogonin-induced apoptosis was validated by AO/EB staining which showed remarkable changes in the nuclear morphology and membrane blebbing of the SW48 cells (Figure 6). The apoptosis was further confirmed by the increased expression of Caspase 3, 8, 9 and Bax and decreased expression of the Bcl-2 in SW48 cells (Figure 7).

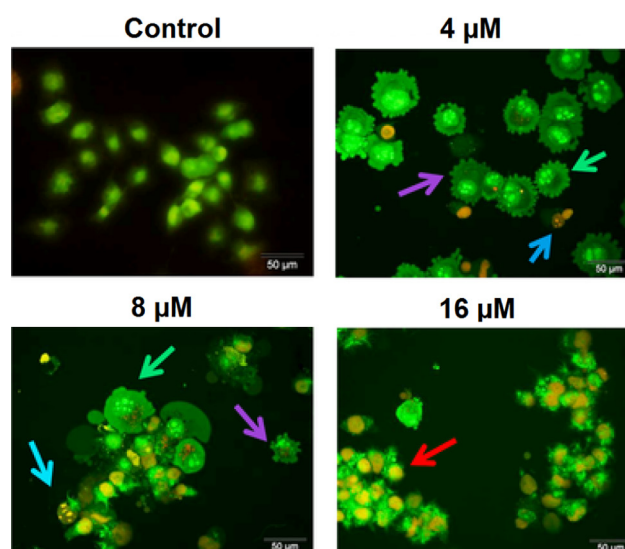


Figure 6. Wogonin triggers apoptosis in SW48 colorectal cancer cells as depicted by AO/EB staining. The experiments were performed in triplicate. Green arrow indicates early apoptotic cells; blue arrow shows late apoptotic cells; purple arrow shows membrane blebbing; and red arrow shows loss of membrane shape.

Wogonin causes the G2/M arrest of colorectal cancer cells

The effects of wogonin on the distribution of SW48 cells in various cell cycle phases was assessed by means of flow cytometry which showed that wogonin caused remarkable increase in the percentage of the SW48 cells in the G2 phase of the cell cycle. The percentage of SW48 cells in the

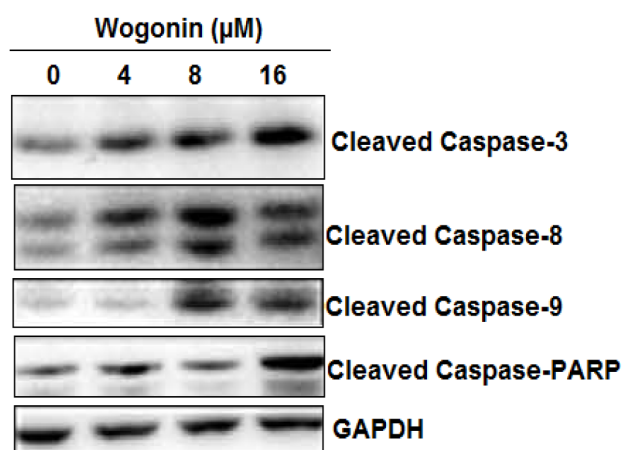


Figure 7. Effect of wogonin on the apoptosis-related protein expression as depicted by western blotting. The Figure shows that the expression of cleaved caspase-3, 8 and 9 and cleaved PARP increases with increase in the concentration of wogonin. The experiments were performed in triplicate.

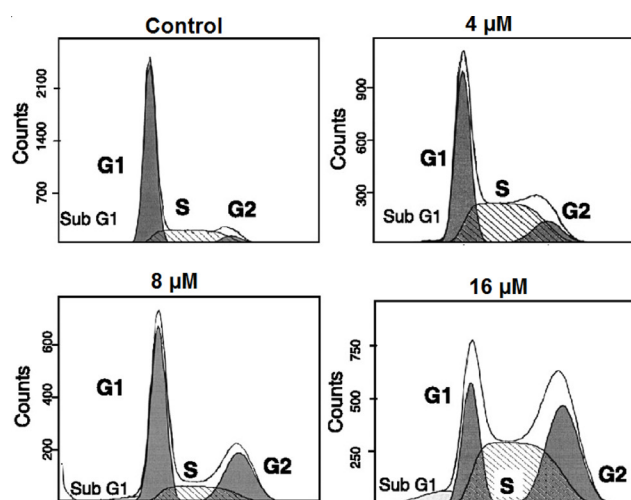


Figure 8. Wogonin triggers G2/M cell cycle arrest in the SW48 cells in a concentration-dependent manner as depicted by flow cytometry. The experiments were performed in triplicate.

G2 phase increased from 10.12% to 48.15% upon treatment with wogonin (Figure 8). These results clearly indicate that wogonin induces G2/M cell cycle arrest of the colorectal cancer cells.

Wogonin inhibits the PI3K/AKT and STAT3 signalling pathway

Next, we sought to know the effects of wogonin on the PI3K/AKT and STAT3 signalling pathway of SW48 colorectal cancer cells. It was revealed that wogonin caused concentration-dependent decline in the phosphorylation of p-PI3K and p-AKT, while no apparent effect was observed on the expression of total PI3K and AKT (Figure 9A). Similarly, the phosphorylation of p-STAT3 (Tyr 705) and p-STAT3 (Ser 727) was significantly decreased while the total STAT3 remained unaltered (Figure 9B).

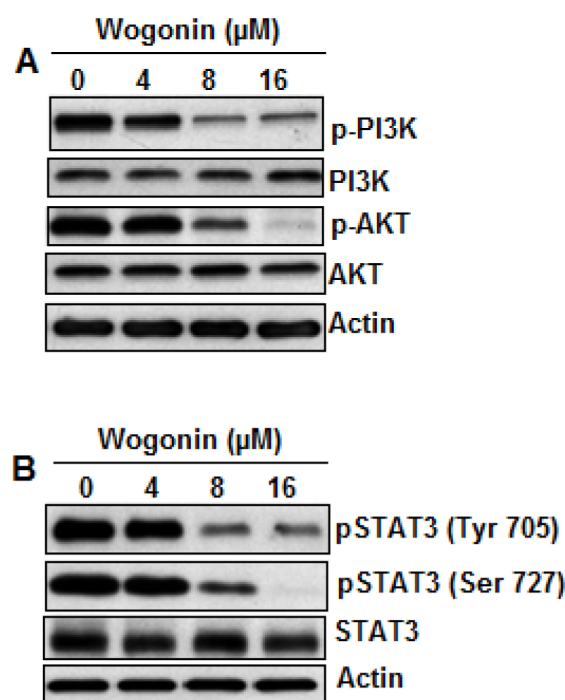


Figure 9. Wogonin inhibits **A:** PI3K/AKT and **B:** STAT3 signalling pathways as depicted by western blot analysis. The Figure shows that wogonin blocks the PI3K/AKT and STAT3 pathway in a concentration-dependent manner. The experiments were repeated in triplicate.

Discussion

Colorectal cancer is a devastating malignancy and its incidence is expected to increase dramatically in the coming years [13]. The clinical outcome is not satisfactory and treatment strategies have a number of flaws. The currently available chemotherapeutic agents have adverse effects, sometimes dangerous. Besides, emergence of chemoresistance in cancer cells further makes it difficult to treat colorectal cancer [3]. Herein, we report that a natural flavone wogonin from *Scutellaria baicalensis* exerts antiproliferative effects on human colorectal cancer cells. The anticancer effects were dose-dependent and were also confirmed by the results of colony formation assay. Previous studies have also reported the anticancer potential of wogonin. Wogonin has been reported to inhibit the growth of osteosarcoma, breast cancer and cervical carcinoma, to name a few [14-16]. Furthermore, it was observed that wogonin exhibits limited cytotoxicity on the normal cells, suggesting that wogonin acts specifically on the cancer cells. To gain insights about the mechanism of action, electron microscopic studies were performed on the wogonin-treated SW48 colorectal cancer cells and the results showed that wogonin causes the formation autophagic vacuoles and the wogonin-induced autophagy was confirmed by

western blot analysis which showed upregulation of LC3II and Beclin-1 expression. Autophagy is a degradation process that eliminates the defective proteins and organelles and plays an important role in the inhibition of tumorigenesis [17]. We also investigated if wogonin triggers apoptotic cell death as well. The results of AO/EB staining showed that wogonin induces membrane blebbing and apoptosis. The apoptosis in SW48 cells was further confirmed by examining the expression of the apoptosis related proteins. Wogonin increased the expression of Caspase 3, 8 and 9 and Bax and decreased the expression of Bcl-2. Apoptosis removes the defective or cancer cells and maintains the tissues homeostasis [18]. Previous studies have also shown that wogonin induces apoptotic cell death in breast cancer and glioblastoma cells [19,20]. Cell cycle arrest also helps to halt the growth of cancer cells [21]. Wogonin has also been reported to trigger cycle arrest in cervical cancer cells [22]. In this study, we found that wogonin blocks the

cells at the G2/M checkpoint. Finally, the effects of wogonin were also investigated on the PI3K/AKT and STAT3 signal transduction pathways. These pathways have been shown to be aberrantly activated in cancer cells [23] and wogonin could inhibit both these pathways in the SW48 colorectal cancer cells, suggestive of its potential anticancer effects.

Conclusion

Wogonin exerts considerable anticancer effects on the human colorectal cancer cells by induction of autophagy and apoptosis. Besides wogonin also triggers G2/M cell arrest in the colorectal cancer cells. Hence, wogonin may prove a potential lead molecule and merits further investigations.

Conflict of interests

The authors declare no conflict of interests.

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