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ORIGINAL ARTICLE

Norwogonin flavone suppresses the growth of human colon cancer cells via mitochondrial mediated apoptosis, autophagy induction and triggering G2/M phase cell cycle arrest

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Summary

Purpose: *Colorectal cancer is one of the deadly malignancies and is one of the top three most common cancers and the third leading cause of cancer-related deaths. The main objective of the study was to investigate the anticancer effects of norwogonin - a naturally occurring plant flavone. We also examined its effects on programmed cell death, autophagy and cell cycle phase distribution.*

Methods: *Cell viability of colon cancer cells was evaluated by MTT assay while apoptotic studies were carried out by fluorescence microscopy using acridine orange (AO)/ethidium bromide (EB) and Comet assays. Transmission electron microscopy (TEM) was used to study formation of autophagosomes reminiscent of autophagy. Furthermore, western blot assay was used to study the effects of norwogonin on apoptosis-related protein expressions including Bax, Bcl-2 and autophagy-related proteins. Effects on cell cycle were evaluated by flow cytometry.*

Results: *The results showed that norwogonin causes substantial reduction in the viability of the human colorectal carcinoma cells in a dose-dependent manner, exhibiting an IC_{50} of 15.5 μM in cancer cells and IC_{50} of 90 μM in normal cell lines. The AO/EB staining assay showed that norwogonin suppresses the viability of cancer cells via induction of apoptotic cell death which was associated with increase in Bax and decrease in Bcl-2 levels. Comet assay results also confirmed that norwogonin induces apoptosis. Norwogonin also led to induction of autophagy along with triggering G2/M phase cell cycle arrest.*

Conclusions: *In conclusion, the current study shows that norwogonin has a potential to inhibit in vitro colorectal cancer cells growth by triggering apoptosis, autophagy and cell cycle arrest and as such could be developed as a possible anticancer agent.*

Key words: *Colorectal cancer, apoptosis, autophagy, cell cycle arrest, flow cytometry*

Introduction

Colorectal cancer (CRC) is counted among the top three most frequent cancers affecting people. It is also the third major cause of cancer-linked mortality in the United States. CRC prevalence and mortality rates in developed countries has been declining during the last 10 years but in developing countries CRC is still a major health problem and is growing all the time, mainly due to population aging and western lifestyle [1,2]. CRC is an aggressive malignancy with poor prognosis. CRC cells are difficult to target as these cells have a strong tendency to avert apoptosis and have also strong ability to invade and migrate to neighbouring tissues and as such have a strong metastasis tendency [3,4]. Con-

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cerning the treatment of CRC, surgical resection is the main treatment option performed in patients with early-stage disease. However, for advanced stages, surgical resection is often accompanied by chemotherapy as well as radiotherapy. Currently, for treating primary colon carcinoma, 5-fluorouracil alone or in combination with oxaliplatin or folinic acid are prescribed. However, for advanced CRC, a combination of irinotecan with 5-fluorouracil or folinic acid is the principal chemotherapy regimen used. Despite the fact that chemotherapy and radiotherapy for CRC are effective, they are associated with adverse side-effects like neurotoxicity, hair loss, vomiting, suppression of immune system, and affecting the patient quality of life. This is further complicated by the fact that CRC cells are increasingly becoming resistant to chemotherapeutic agents, rendering chemotherapy less efficacious [5-7]. Emergence of targeted therapies like bevacizumab or cetuximab are the alternative treatments for CRC, but despite enhancing the survival rates of CRC patients, the cost for such treatments is beyond the reach of an ordinary individual and is practically unaffordable [8]. Therefore, there is a pressing need for novel compounds which are more effective, cheaper and are devoid of adverse side effects. Plants synthesize a wide spectrum of bioactive molecules with potent anticancer activities. The majority of the currently used anticancer drugs in the clinic originate from natural sources, especially plants [9]. Therefore, the main objective of the current research work was to examine the anticancer activity of norwogonin flavone in human CRC cells along with studying its effects on apoptosis, autophagy and cell cycle phase distribution.

Methods

Cell lines, culturing conditions and cell viability

The SW48 human colon adenocarcinoma cell line and CCD-18Co normal colon fibroblast cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) at 37°C with 98% humidity and 5% CO_2. The cytotoxic effect of norwogonin was assessed on colon cancer and normal cell line by MTT assay as previously described by Mosmann [10]. The colon cancer cells were treated with increasing concentrations of norwogonin and the proliferation rate was determined by taking absorbance at 570 nm using a spectrophotometer (BioTek, USA). The cell viability was obtained from the absorbance data.

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

The SW48 human colon cancer cells at a cell density of 1x10^6 cells/ml were placed into 6-well plates and grown for 24 h. The cells were exposed to treatment with increasing concentrations of norwogonin and incubated for 24 h. Afterwards, 30 µl of cell culture were put onto glass slides and stained with AO/EB separately. The slides were then covered with cover slips and examined under fluorescence microscope (Nikon Instruments Inc., NY, USA). Comet assay, which measures the DNA damage, was carried out by alkaline single cell gel electrophoresis according to the guidelines of the method formerly published in the literature [11,12].

Transmission electron microscopy

In order to examine whether norwogonin induces autophagic effects in SW48 human colon cancer cells, transmission electron microscopy (TEM) was carried out. In brief, SW48 cells were subjected to increasing doses of norwogonin for 24 h, were then collected using trypsinization and washed twice with phosphate buffer saline (PBS). The cells were then fixed in 2% glutaraldehyde having 0.1 M phosphate buffer. Using 1.5% solution of osmium tetroxide, the cells were post-fixed and after that the cells were treated with ethyl alcohol and then embedded in resin. Thin sections were cut with ultramicrotome and then observed by means of a Zeiss CEM 902 electron microscope.

Flow cytometric analysis

The SW48 human colon cells (at a cell density of 1x10^5 cells/ml) cultured in DMEM (Gibco, USA)

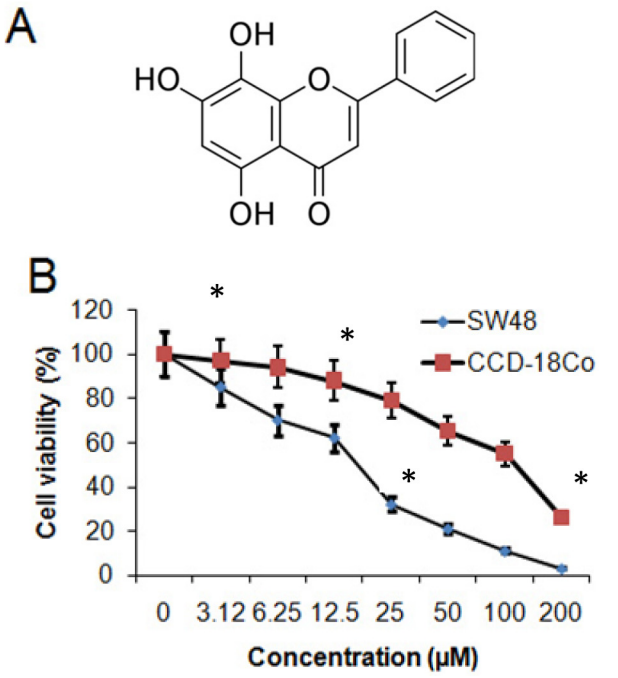

Figure 1. A: Chemical structure of Norwogonin. **B:** Cell viability assay showing the inhibitory effects of norwogonin on the viability of the SW48 human colon carcinoma cells along with normal colon cell line (CCD-18c). The experiments were executed in triplicate and presented as mean ± SD (*p<0.05).

were treated with increasing concentrations of norwogonin flavone harvesting, the cells were fixed with ice-cold ethanol and then treated with RNase A (10 µg/ml). Then, the cells were washed twice with PBS and then, using 30 µl solution of propidium iodide (PI) stain, the SW48 cells were stained. Using a FACSCalibur flow cytometer (FACSCalibur; BD Biosciences, New York, USA), the distribution of the cancer cells in different phases of the cell cycle was determined.

Western blot analysis

The SW48 human colon cells were initially washed with ice-cold PBS and then lysed in RIPA lysis buffer containing the protease inhibitor. The protein content of each lysate was estimated by bicinchoninic acid (BCA) assay. The samples were then loaded on the SDS-PAGE and the gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for 24 h. After this, the membranes were incubated with HRP-conjugated secondary antibody (1:1000) for 50 min at 25°C. Superior chemiluminescence reagent was used to analyze the protein bands. Lastly, the signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalisation.

Statistics

The experiments were done in triplicate and the values shown are the mean of three experiments±SD. $P<0.05$ was considered statistically significant. Student's t-test using GraphPad prism 7 software was employed for statistical analyses.

Results

Norwogonin induced dose-dependent and selective cytotoxicity in SW48 human colon cancer cells

The MTT cell viability assay which was performed to evaluate the antiproliferative effects of norwogonin indicated that this molecule at tested doses of 0, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM exerted significant and concentration-dependent cytotoxicity. The structure and cell viability data are shown in Figure 1A and 1B, respectively. Interestingly, the cytotoxicity of norwogonin against CCD-18Co normal colon fibroblast was much less pronounced, indicating that norwogonin is much less toxic to normal cells as compared to colon cancer cells.

Norwogonin led to DNA damage and cell apoptosis

In order to investigate the effects of norwogonin on the DNA damage and cell apoptosis, fluorescence microscopy was employed for Comet assay and AO/EB staining assay. Figure 2 shows the Comet assay results which indicate that, as compared to the untreated control cells which showed intact DNA without any fragmentation, norwogonin-treated cells showed significant fragmented DNA. The DNA-fragmentation in these cells was concentration-dependent as can be easily seen from longer comet tails at higher doses.

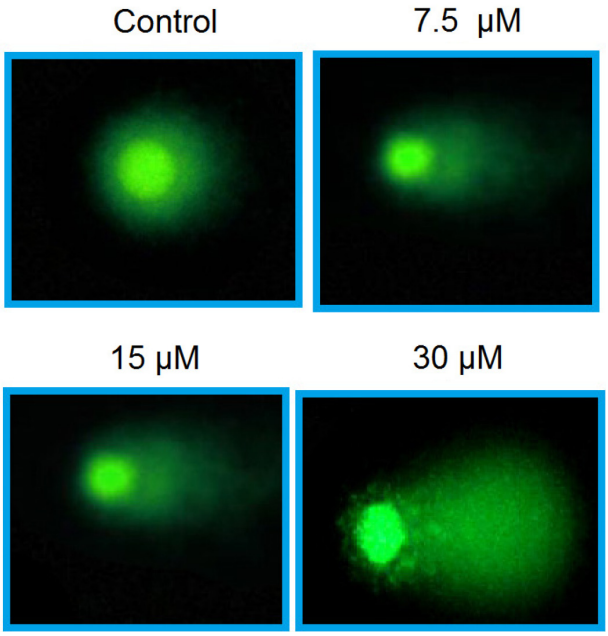

Figure 2. Comet assay using fluorescence microscopy showing the effects of norwogonin on the DNA fragmentation in human colorectal cancer cells. The results showed increasing DNA fragmentation with increased in norwogonin dose. The experiments were performed in triplicate.

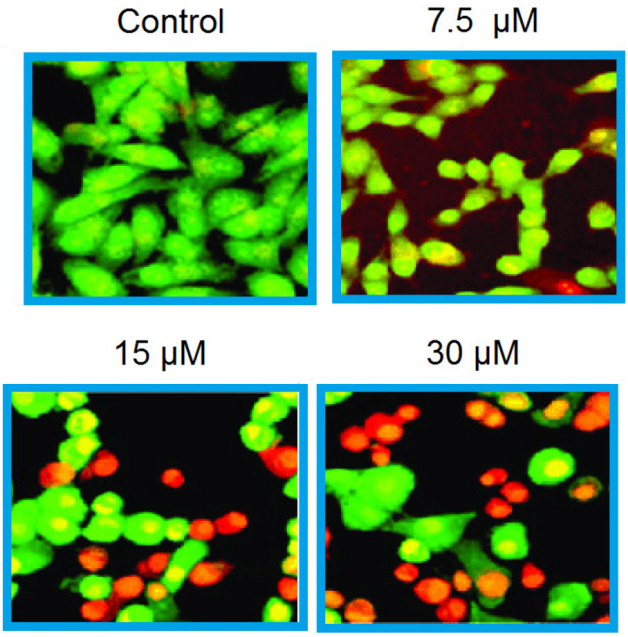

Figure 3. Acridine orange/ethidium bromide (AO/EB) staining assay using fluorescence microscopy indicating norwogonin induces concentration-dependent apoptosis in human colorectal cancer cells. The experiments were performed in triplicate.

Figure 3 shows the results of AO/EB staining results indicating that with increasing doses of norwogonin, the number of cells with red/orange fluorescence increases drastically, suggestive of apoptosis. Untreated control cells showed no signs of apoptosis as no cells with orange fluorescence could be seen. In order to confirm the results that the molecule indeed induces apoptosis western blot assay was performed to analyse the effects on Bax and Bcl-2 protein expressions. The results showed that norwogonin led to increase in Bax expression and successive decrease in Bcl-2 expression dose-dependently (Figure 4).

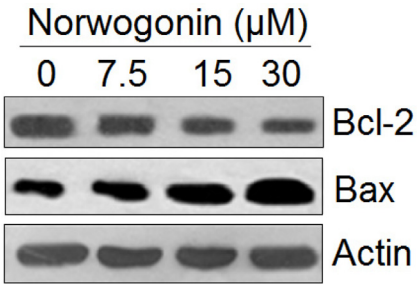

Figure 4. Evaluation of the effect of norwogonin on the expression levels of Bax and Bcl-2 (apoptosis-associated proteins) using western blotting Norwogonin led to decreased Bcl-2 expression and to increased Bax expression. The experiments were performed in triplicate.

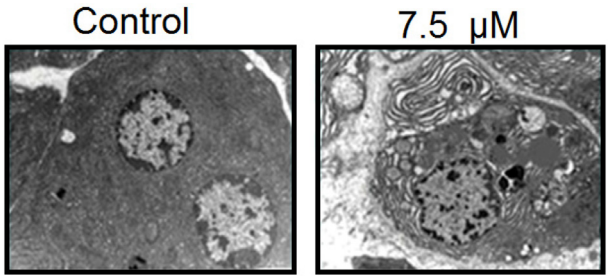

Figure 5. Transmission electron microscopy shows the autophagic effects induced by norwogonin in human colorectal cancer cells. The experiments were performed in triplicate.

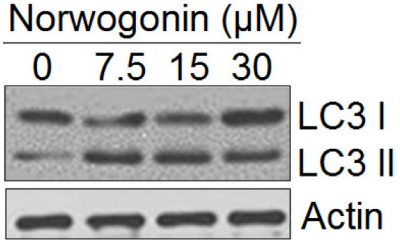

Figure 6. Evaluation of the effect of norwogonin on the expression levels of LC3-I and LC3-II (autophagy-related proteins) using western blot method. Norwogonin led to increase in LC3-I and LC3-II expression, indicating autophagy. The experiments were performed in triplicate.

Norwogonin also induced autophagy in SW48 human colon cancer cells

TEM was employed to investigate whether norwogonin could induce autophagy in SW48 cells in addition to inducing apoptosis that were seen in above results. The results of TEM are depicted in Figure 5 and show the appearance of autophagosomes at 7.5 μM dose of the molecule. There was a direct link between onset of autophagosomes and autophagy process. At zero dose of norwogonin, no such cellular structures were observed. Western blot assay was further used in order to authenticate the results of TEM. The results indicated that norwogonin treatment led to dose-dependent increase of LC3-II and LC3-I expressions hinting towards autophagic cascade. Western blot results are shown in Figure 6.

Norwogonin induced G2/M cell cycle arrest in SW48 human colon cancer cells

Flow cytometry was utilised using PI as staining agent in order to study the effects of norwogonin on the cell cycle phase distribution. The results (Figure 7) depict that norwogonin induced dose-dependent G2/M cell cycle arrest, as compared to the untreated control cells with 4.5% cells in G2/M phase, showing the percentage of G2/M phase cells increased to 20.1% at 30 μM concentration of norwogonin. Also, the effects of norwogonin on the expression of cyclin B1 showed that with increasing dose of the molecule, the expression of cyclin B1 decreased dose-dependently (Figure 8).

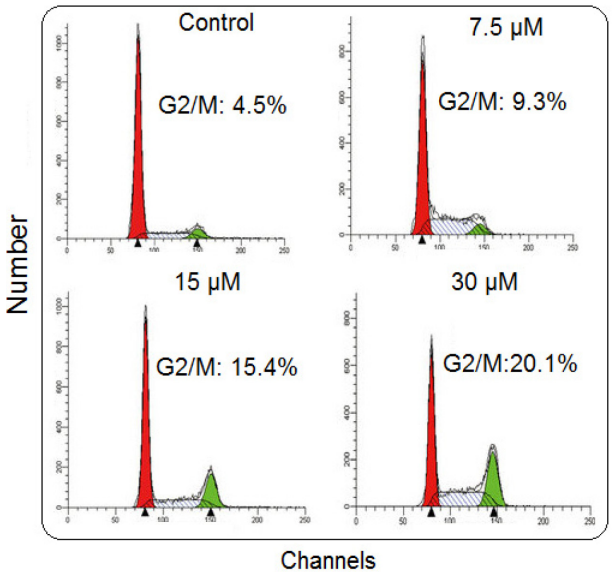

Figure 7. Effects of norwogonin on the cell cycle phase distribution in human colorectal cancer cells using flow cytometry. Increasing doses of norwogonin led to a dose-dependent G2/M phase cell cycle arrest. The experiments were performed in triplicate.

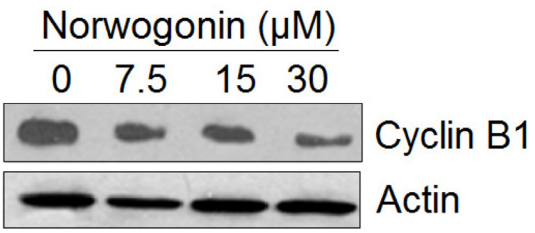

Figure 8. Evaluation of the effect of norwogonin on the expression levels of cyclin-B1 (cell cycle-related proteins) using western blot method. Norwogonin led to decrease in cyclin B1 expression, showing its effect on the cell cycle. The experiments were performed in triplicate.

Discussion

Apoptosis is a well organised biochemical process which helps eradicate the cells with damaged DNA and cytoskeleton. Apoptosis is perhaps the most well studied biological process as this process plays pivotal roles in both normal and pathological conditions. It is also known as programmed cell death which is characterized by certain morphological and biochemical changes within the cells. These changes include caspase activation, DNA fragmentation and protein degradation, cell membrane damage, changes in cellular morphology, nuclear disintegration and fragmentation, cellular contraction and membrane blebbing [13,14]. The most prominent feature of apoptosis is the activation of caspases which are actually cysteine proteases that can slice numerous important cell proteins that degrade the nucleus. These caspases can also trigger DNAases which catalyse nuclear DNA damage [15]. Many of the naturally occurring plant secondary metabolites are known which have been reported to induce programmed cell death in a number of cancer cell lines. In the present study, we have investigated the anticancer effects of norwogonin (5,7,8-trihydroxyflavone) against SW48 human colon carcinoma cells along with a normal colon cell line (CCD-18co). Norwogonin has been reported to exhibit anticancer effects in numerous cancer cell lines. Some authors report anticancer effects of norwogonin against human triple-negative breast cancer cells via downregulation of Nf-kB, STAT3 and TAK1 signalling pathway [16]. Norwogonin has also been reported to induce differential apoptotic effects in human leukemia cells via ROS stimulation [17]. However, the anticancer effects of norwogonin against human colon cancer cells have not been reported so far. The results of the current study revealed that norwogonin induced selective and dose-dependent anticancer effects in human colon cancer cells. The cytotoxicity of norwogonin against CCD-18Co normal colon fibroblast was much less pronounced, indicating that norwogonin is much less toxic to normal cells as compared to colon cancer cells. This molecule also induced DNA damage and cellular apoptosis as indicated by Comet assay, fluorescence microscopy and western blot. The expression of Bax was increased while as the expression of Bcl-2 was shown to decrease with increasing norwogonin dose. Further experiments carried out also indicated that norwogonin not only induces apoptosis but also autophagy which was initially revealed by TEM and then confirmed by western blot assay. Finally, norwogonin was also shown to induce G2/M cell cycle arrest with concomitant decrease of cyclin-B1 expression.

Conclusions

In conclusion, these results indicate that norwogonin has a strong anticancer activity in human colon cancer and its anticancer effects are mediated via induction of apoptosis, autophagy and G2/M cell cycle arrest.

Conflict of interests

The authors declare no conflict of interests.

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