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ORIGINAL ARTICLE

Naturally occurring glycyrrhizin triterpene exerts anticancer effects on colorectal cancer cells via induction of apoptosis and autophagy and suppression of cell migration and invasion by targeting MMP-9 and MMP-2 expression

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Summary

Purpose: *The main aim of the current study was to investigate the anticancer properties of naturally occurring triterpene – glycyrrhizin - against human colorectal carcinoma cells along with evaluation of its effects on cells apoptosis, autophagy and cell migration and invasion.*

Methods: *Cell viability was evaluated by CellTiter95® Aqueous One Solution cell viability assay, while the effects on cell apoptosis were observed by fluorescence microscopy using DAPI staining. Effects on autophagy were detected by transmission electron microscopy (TEM) along with western blot method. Transwell assay was performed to monitor the effects on cell migration and invasion.*

Results: *Glycyrrhizin induced selective and dose-dependent inhibition of cell growth in SW48 human colorectal carcinoma cells with lesser cytotoxicity in normal colon cells (CCD-18Co). Glycyrrhizin also led to cell apoptotic effects manifested by chromatin condensation and nuclear fragmentation as evidenced by brighter fluorescence. Apoptosis was confirmed by western blot which showed increase in Bax expression and decrease in Bcl-2 expression. TEM analysis showed that glycyrrhizin-treated cells at 12 μM showed autophagosomes indicating onset of autophagy. Western blot assay confirmed the autophagy results which showed glycyrrhizin-treated cells indicated increased expression of Beclin-1, LC3B-I and LC3B-II in a dose-dependent manner. Glycyrrhizin treatment also led to inhibition of both cell migration and invasion.*

Conclusion: *The results of this study reveal that glycyrrhizin can be developed as a potent anticancer agent against colorectal cancer provided further studied are performed, especially on its toxicity to humans.*

Key words: *glycyrrhizin, colorectal cancer, apoptosis, autophagy, cell invasion*

Introduction

Colorectal carcinoma (CRC) is the most abundant type of cancer in both males and females, ranking third and second, respectively. About 1.24 million new cases of CRC were registered worldwide in 2008 alone, indicative of CRC high incidence [1]. According to a study by Canadian Cancer Society, the incidence rate of CRC among all cancer types is third highest in both genders [2]. Countries which have maximum rate of incidence of CRC include North America, Oceania, and Europe, while the countries with minimum rate of incidence of CRC include Africa and some central and south Asian countries [3]. Among all malignancies diagnosed in Saudi Arabia, CRC ranked first in males and third in females in 2011 [4]. The incidence of colon carcinoma is primarily linked with the oc-

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currence of ACF (abnormal crypt foci), an initial neoplastic lesion. ACF are bunches of mucosal cells with a thicker and inflated layer of epithelia than the normal neighbouring crypts, which develop into polyps then adenomas and later adenocarcinomas [5]. These steps are considered to be the result of accretion of multiple somatic mutations in colonic epithelium (about 70%) [6,7]. However, all the ACF don't develop into colon carcinoma, yet various studies have described that all colon cancers originate from ACF [5]. There are several types of inherited CRC including familial adenomatous polyposis (FAP), hereditary nonpolyposis colorectal cancer (HNPCC), hamartomatous polyps as primary lesions in juvenile polyposis syndrome (JPS) and Peutz-Jeghers syndrome (PJS), attenuated FAP, hyperplastic polyposis (HPP), MUTYH-associated polyposis (MAP) and familial CRC (FCC) syndrome X [8-10]. Smoking (more than 20 cigarettes per day), alcohol consumption and inflammatory bowel disease are the main causes associated with CRC [11-13]. In United States, CRC is the third prominent cause of cancer related deaths and the 5-year overall survival (OS) of this malignancy is around 65%. Thus to curb this malignancy and improve OS, novel approaches and methodologies are the need of the hour. In the recent past, natural products have been explored for their pharmacologic importance, being used for treatment of many diseases from early human era, including their anticancer potential. Glycyrrhizin belongs to triterpene glycosides, and is the key constituent of licorice (Glycyrrhiza glabra Linn.). Due to diverse pharmacologic properties of licorice it is used as herbal medicine. Glycyrrhizin has been shown to exhibit anticancer, antiproliferative, antiinflammatory, antioxidant, and antiviral properties [14,15]. This molecule has been reported to exhibit anticancer activity against a range of human cancer cell lines [16-19].

In the current study we tested glycyrrhizin for its anticancer effects on colorectal cancer cells and we observed that anticancer effects were mediated via induction of apoptosis, autophagy and suppression of cell migration and invasion by targeting metalloproteinase (MMP)-2 and -9 expression.

Methods

Cell viability detection

To quantify the rate of cell proliferation of normal colorectal cells (CCD-18Co) and colorectal cancer cells (SW48), cells were seeded at a density of 1×10^4 cells in 96-well plates and incubated for 24 h. After incubation, these cells were treated with varying concentrations of glycyrrhizin (0, 1.25, 5, 10, 20, 40, 80, 160 and 320 μM). MTS (CellTiter 96® AQueous One Solution Cell viability Assay) assay was performed in accordance with the manufacturer's guidelines (Promega, Madison, WI). Cell viability was assessed by using a microplate reader and at 490 nm of absorbance.

Apoptosis detection by DAPI

For the determination of apoptosis induction in SW48 human CRC cells by glycyrrhizin, cells were grown in 6-well plates, followed by incubation at 37°C for 12 h. Subsequently, target cells were exposed to glycyrrhizin treatment at varying doses (0, 10, 20, and 40 μM) for 24 h at 37°C. After the SW48 cells started to cast-off, about 15 μM of cast cells were collected and put on glass slides. Further, these cells were stained with DAPI (4',6-diamidino-2-phenylindole), a fluorescent dye. Finally, cover slip was used to protect the cells on glass slides and observed under a fluorescent microscope.

Transmission electron microscopy for autophagy detection

The effect of autophagy induction by glycyrrhizin on SW48 CRC line was assessed by transmission electron microscopy (TEM). Briefly, SW48 cells were treated with glycyrrhizin at different concentrations (0 and 12 μM) for 12 h. Subsequently, trypsinization was performed for cell assembling, which was followed by washing with phosphate buffered saline (PBS). After washing target cells were fixed with 2% glutaraldehyde in 0.1M phosphate buffer, followed by post fixation with 1% osmium tetroxide (OsO_4). Afterwards, the post fixed cells were exposed to ethanol and embedded in resin. Then, thin and tinny sections were cut using ultramicrotome and observation was done under electron microscope.

Transwell migration assay for cell migration detection

The anti-migration effect of glycyrrhizin in SW48 cells was determined by using transwell assay. In the upper chambers, 4×10^3 cells were put in 590 μL of RPMI-1640 medium with varying doses of glycyrrhizin and the lower chambers were filled with 790 μL of medium plus 10% fetal bovine serum (FBS). Both chambers were incubated at 37°C for one and a half day. After incubation, cleaning of the upper chamber was done with a cotton swab to wash out the non-migrated cells. After that the migrated cells were fixed in 4% formalin for 15 min, followed by staining with 0.1% crystal violet for 20 min. Later pictures of different fields were captured at control, 10, 20 and 40 μM using microscopy at 100x magnification.

Cell invasion assay

The ability of glycyrrhizin to suppress SW48 cell invasion was assessed by transwell chamber assay with Matrigel. Around 250 ml of cultured cells were placed in the upper chamber and only media filled the lower chamber. Afterwards the invaded cells were collected from the upper chamber and incubated for 1 day, followed by fixation with methyl alcohol. After fixation, these cells were stained with crystal violet dye. To calculate the effect on cell invasion by glycyrrhizin in SW48 human CRC cells, an inverted microscope with a magnification of 200x was used.

Western blotting analysis

To evaluate the protein expression in glycyrrhizin-treated SW48 CRC cells western blotting was used (treated at different doses 0, 10, 20 and 40 μM). These cells were first lysed with lysis buffer (RIPA buffer) and bicinchoninic acid assay (BCA assay) was used for assessment of protein content inside each lysate. In brief, using SDS-PAGE, about 10-30 μg of sample proteins were separated and transported to PVDF membranes. These membranes were probed with primary antibodies followed by incubation. Thereafter, these membranes were subjected to horseradish peroxidase-conjugated secondary antibodies treatment and the signal was spotted using a chemiluminescence reagent kit (Amersham Pharmacia, Piscataway, NJ).

Statistics

All the experiments were performed in triplicate, and the data were presented as mean±standard deviation. SPSS version 17 was used to perform the statistical analyses, and the results were analysed using Student's t-test and one-way ANOVA. P<0.05 was considered statistically significant.

Results

Glycyrrhizin inhibited the cell proliferation of SW48 colorectal carcinoma cells

The antiproliferative effect of glycyrrhizin (Figure 1A) on SW48 human CRC cells was evaluated by MTS assay. This assay revealed that the glycyrrhizin-treated cells showed significant decrease in viability concentration-dependently, as compared to the normal CCD-18Co colorectal cells. The cell viability of cancer cells decreased from 100% to 10% with increasing doses of the test molecule (0, 1.25, 5, 10, 20, 40, 80, 160 and 320 μM), while the viability of normal cells remained almost unchanged at lower doses, thus indicating selectivity of glycyrrhizin on inducing antiproliferative effects against CRC cells. The IC_{50} of the glycyrrhizin was 12 μM in SW48 cells and 45 μM in CCD-18Co cells after 24 h of exposure (Figure 1B).

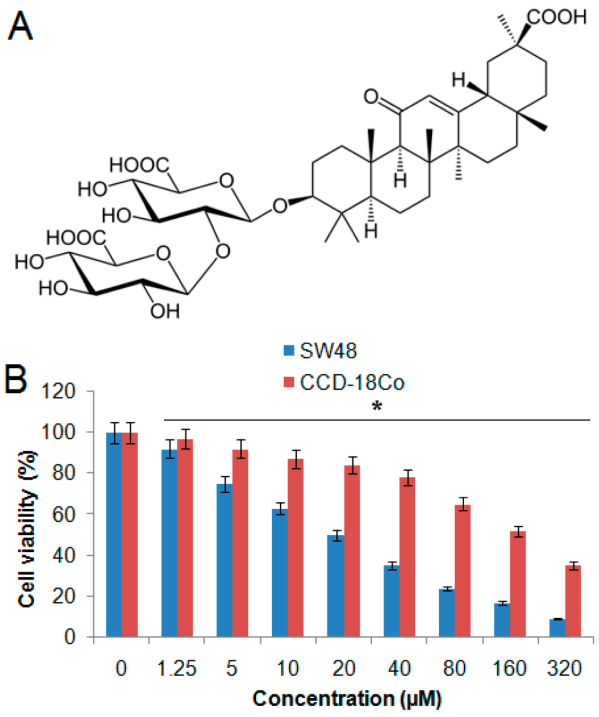

Figure 1. A: Structure of Glycyrrhizin. **B:** MTS assay showing the effects of Glycyrrhizin on the viability of SW48 and CDD-18Co cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Glycyrrhizin resulted in death of colorectal cancer cells via apoptosis induction

The effect of apoptosis induction in SW48 CRC cells by glycyrrhizin was evaluated through fluorescence microscopy using DAPI staining. Images were captured at different intervals with different doses of the molecule and it was observed that the rate of apoptosis increased significantly concentration-dependently (control, 10, 20 and 40 μM) (Figure 2). Glycyrrhizin-treated cells showed a dislocated, fragmented, condensed and shrunken nucleus, high fluorescence, and the chromatin material was randomly distributed as compared to untreated cells which showed smooth, flat, normal nucleus with normally distributed chromatin and low fluorescence. Further, western blotting analysis was used to measure the expression of proteins involved in apoptosis, which revealed that exposure of SW48 CRC cells to the test molecule

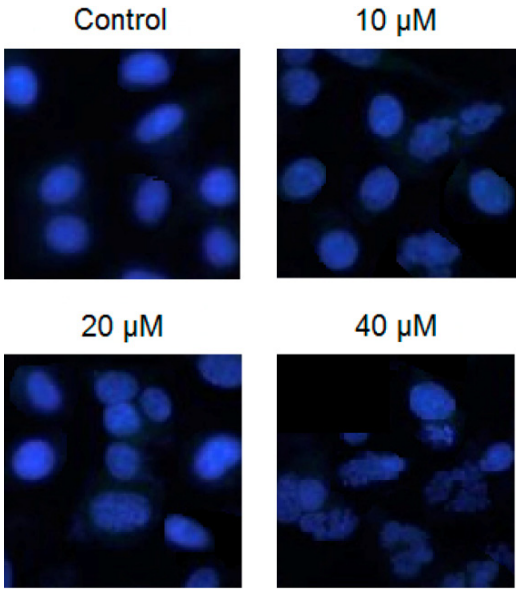

Figure 2. DAPI staining showing dose-dependent induction of apoptosis in SWE48 cells by Glycyrrhizin. The experiments were performed in triplicate.

increased the expression of BAX and decreased the expression of BCL-2 (Figure 3). This is clearly an indication of apoptosis induction by the given drug.

Autophagy induction by Glycyrrhizin

Next, to analyse the effect on autophagy induction by glycyrrhizin in the SW48 CRC cells we performed transmission electron microscopy (TEM). TEM revealed that the treated cells showed formation of autophagosomes or autophagic vesicles which are clearly suggestive of autophagy (Figure 4). In addition, western blotting analysis revealed that treated cells also showed upregulation of autophagy-associated proteins (Beclin-1, LC3B-I and LC3B-II) in a dose-dependent manner (Figure 5).

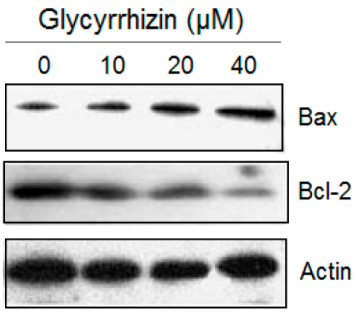

Figure 3. Western blot analysis showing increase in Bax and decrease in Bcl-2 expression in a dose-dependent manner upon Glycyrrhizin treatment. The experiments were performed in triplicate.

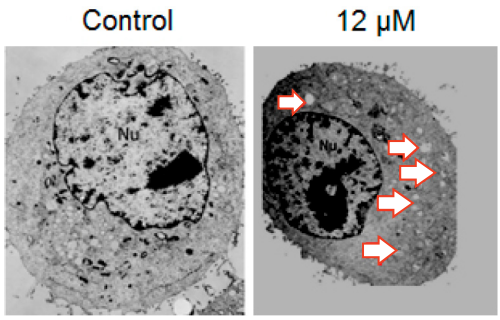

Figure 4. TEM analysis showing induction of autophagy in SWE48 cells by Glycyrrhizin (arrows depict autophagosomes). The experiments were performed in triplicate.

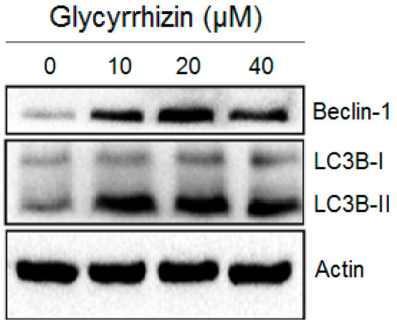

Figure 5. Western blot analysis showing increase in LC3B-II and Beclin-1 and no change in LC3B-1 expression in a dose-dependent manner upon Glycyrrhizin treatment. The experiments were performed in triplicate.

Inhibition effect of Glycyrrhizin on cell migration and cell invasion of SW48 human colorectal carcinoma cells

To evaluate the effect of glycyrrhizin on cell migration and invasion of SW48 CRC cell line we performed transwell cell migration and invasion assays respectively. The results revealed that both cell migration (Figure 6) and invasion (Figure 7) of cancer cells were supressed or declined significantly by the exposure to varying concentrations (0, 10, 20 and 40 μM) of the molecule, indicating dose-dependent inhibition of cell migration and invasion of SW48 cancer cells.

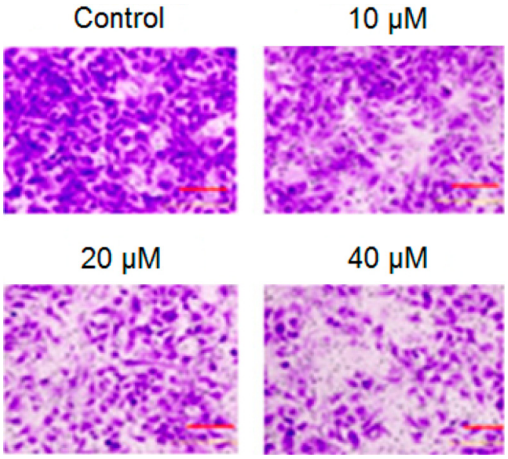

Figure 6. Transwell assay showing decrease in the migration of the SW48 cells in a dose-dependent manner upon Glycyrrhizin treatment. The experiments were performed in triplicate.

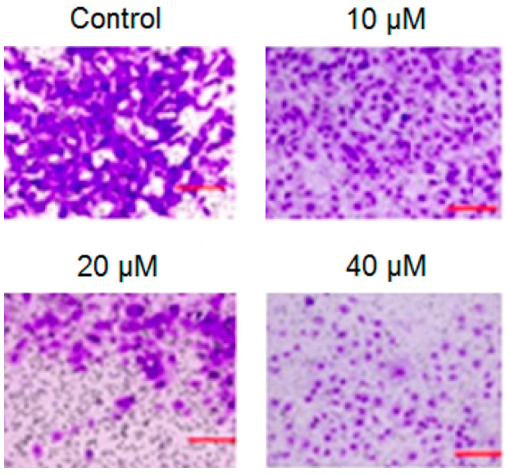

Figure 7. Transwell assay showing decrease in the invasion of the SW48 cells in a dose-dependent manner upon Glycyrrhizin treatment. The experiments were performed in triplicate.

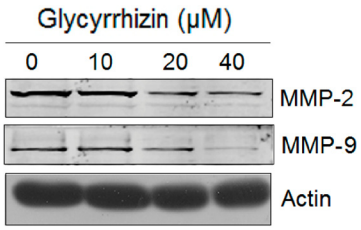

Figure 8. Western blot analysis showing decrease in MMP-2 and MMP-9 in a dose-dependent manner upon Glycyrrhizin treatment of SW48 cells. The experiments were performed in triplicate.

Inhibition of MMP-9 and MMP-2 expression by Glycyrrhizin

MMP-2 and MMP-9, also known as gelatinase-A and gelatinase-B, belong to proteins of the MMP (matrix metalloproteinase) family and are involved in the breakdown of ECM (extracellular matrix) in normal processes. The results revealed that the expression of these two proteins was decreased significantly by glycyrrhizin in a dose-dependent manner (Figure 8).

Discussion

CRC is a life-threatening malignancy worldwide. Its screening is an effective strategy towards early diagnosis and prevention of the disease. Screening procedures include digital rectal examination, barium enema, stool occult blood testing and colonoscopy. Among screening procedures colonoscopy is the most prescribed method and often suggested as a first-line screening approach [20,21]. Primary recognition of CRC may result in surgical treatment only, whereas advanced, late-stage and/or metastasized CRC involve supplementary chemotherapy and radiotherapy. Search for more effective but less toxic new antitumor and chemotherapeutic agents has drawn great attention in phytochemicals due to the side effects, therapeutic limitations, and high cost of conventional medications. Glycyrrhizin, a licorice-derived compound, has been identified with its wide range of pharmacological activities (antiinflammatory, antitumor, antioxidant, as well as antiviral) [22,23]. Herein, we studied glycyrrhizin for its anticancer effects on CRC cells via induction of apoptosis, autophagy and suppression of cell migration and invasion by targeting MMP-9 and MMP-2 expression. To evaluate the anticancer effects of glycyrrhizin in SW48 human CRC cell line we performed a number of assays. Firstly, we performed MTS assay for the determination of cell viability which showed that exposure to the test molecule resulted in significant decreased viability of cancer cells, without affecting that much the normal colorectal cells. In addition, it was observed that inhibition of cell proliferation by the test molecule was dose-dependent. These findings are in agreement with a previous study wherein glycyrrhizin has been reported to suppress the growth of human lung cancer cells [24]. Further, we performed fluorescence microscopy with DAPI staining to evaluate the effect of apoptosis induction by glycyrrhizin in SW48 CRC cells which showed that increasing doses of the test molecule resulted in enhancing the rate of apoptosis. These observations are consistent with a previous study where glycyrrhizin has been reported to induce ROS-dependent apoptosis in human cervical HeLa cancer cells [25]. This was further studied through western blotting analysis by obtaining the expression of proteins related to apoptosis. Bax and Bcl-2 are important biomarker proteins for induction of apoptosis. During induction of apoptosis, the expression of Bax increases and of Bcl-2 decreases [26].

In the present study it was found that glycyrrhizin caused significant increase in the expression of Bax and simultaneous significant decrease in the expression of Bcl-2.

Afterwards, we performed TEM to detect autophagy induction by glycyrrhizin, which showed that exposure of CRC cells to the test molecule resulted in autophagosome formation which was further supported by western blotting analysis that revealed that exposure to the test molecule increased the expression of Beclin-1, LC3B-I and LC3B-II.

Lin et al [27] have reported that glycyrrhizic acid induces human MDA-MB-21 breast cancer cells death and autophagy via ROS-mitochondrial pathway, further confirming our findings.

Furthermore, transwell assay and cell invasion assay revealed that the test molecule significantly inhibited cell migration and invasion in a dose-dependent manner. Further, the expression of MMPs associated with cell migration and invasion (MMP-2 and MMP-9) declined by the treatment of SW48 human colon carcinoma cells in a dose-dependent manner. The inhibition of cell migration and invasion by glycyrrhizin has also been reported on breast cancer cells [28]. Thus, it is clear that glycyrrhizin is a potential anticancer agent and merits further investigations.

Conclusion

Taken together the results of the current study revealed that the naturally occurring glycyrrhizin triterpene exerts anticancer effects on SW48 CRC cell line via induction of apoptosis,

autophagy and suppression of cell migration and invasion by targeting MMP-9 and MMP-2 expression.

Conflict of interests

The authors declare no conflict of interests.

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