Ursolic acid promotes colorectal cancer cell apoptosis and inhibits cell proliferation via modulation of multiple signaling pathways

JIUMAO LIN1,2*, YOUQIN CHEN3*, LIHUI WEI1,2, ALING SHEN1,2, THOMAS J. SFERRA3, ZHENFENG HONG1 and JUN PENG1,2

1 Academy of Integrative Medicine and 2 Fujian Key Laboratory of Integrative Medicine on Geriatric, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350122, P.R. China; 3 Case Western Reserve University School of Medicine, Rainbow Babies and Children's Hospital, Cleveland, OH 44106, USA

Received June 5, 2013; Accepted July 17, 2013

DOI: 10.3892/ijo.2013.2040

Abstract. The development of colorectal cancer (CRC) is strongly correlated with the aberrant activation of multiple intracellular signaling transduction cascades including STAT3, ERK, JNK and p38 pathways which usually function redundantly. In addition, crosstalk between these pathways forms a complicated signaling network that is regulated by compensatory mechanisms. Therefore, most of the currently used and single-target-based antitumor agents might not always be therapeutically effective. Moreover, long-term use of these agents often generates drug resistance. These problems highlight the urgent need for the development of novel anticancer chemotherapies. Ursolic acid (UA) is a major active compound present in many medicinal herbs that have long been used for the clinical treatment of CRC. Although previous studies have demonstrated an antitumor effect for UA, the precise mechanisms of its tumoricidal activity are not well understood. In the present study, using CRC mouse xenograft model and the HT-29 human colon carcinoma cell line, we evaluated the efficacy of UA against tumor growth in vivo and in vitro and investigated the underlying molecular mechanisms. We found that UA inhibits cancer growth without apparent toxicity. Furthermore, UA significantly suppresses the activation of several CRC-related signaling pathways and alters the expression of critical target genes. These molecular effects lead to the induction of apoptosis and inhibition of cellular proliferation. These data demonstrate that UA possesses a broad range of anticancer activities due to its ability to affect multiple intracellular targets, suggesting that UA could be a novel multipotent therapeutic agent for cancer treatment.

Introduction

Colorectal carcinoma (CRC) is a serious global health problem with over one million new cases and half a million deaths worldwide each year (1). To date, chemotherapy is the main therapeutic approach for invasive and metastatic CRC. Despite recent advances in this area, 5-fluorouracil (5-FU)-based regimens continue to be the standard therapeutic approach for patients with advanced CRC (2). However, due to drug resistance and an unacceptable level of toxicity, 5-FU-based regimens produce objective response rates of <40% (3,4).

The pathogenic mechanisms underlying CRC development are complex and heterogeneous and involve multiple cellular signaling transduction pathways including signal transducer and activator of transcription 3 (STAT3), extracellular regulated protein kinases (ERK), c-Jun NH(2)-terminal protein kinases (JNK) and p38 kinase. STAT3 is a transcription factor that plays an essential role in cell survival and proliferation (5). After activation via phosphorylation by receptor and non-receptor protein tyrosine kinases, STAT3 proteins in the cytoplasm dimerize and translocate to the nucleus where they regulate the expression of critical genes involved in cell proliferation and survival (5,6). Constitutive activation of STAT3 is highly correlated with the development of numerous types of cancer including CRC and commonly suggests a poor prognosis (7-10). MAPK signaling is one of the major cell-survival and proliferation pathways. In mammals, there are three major subfamilies of MAPK, including ERK, JNK and p38. Activation of all MAPK pathways is regulated by a central three-tiered kinase core consisting of MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK; wherein MAP3K phosphorylates MAP2K and activated
MAP2K in turn phosphorylates and activates MAPK (11,12). MAPK signaling is important in intestinal epithelial differentiation (13); however, aberrant activation of MAPK pathway is involved in colon carcinogenesis (14-17).

The molecular pathways described above modulate the expression of key genes involved in the regulation of cell proliferation, apoptosis and angiogenesis and are participants in the processes of induction, progression and metastasis of colorectal carcinoma. Thus, each serves as a potential target for novel chemotherapeutics. It is noteworthy that these signaling pathways usually have functional redundancy. In addition, there is crosstalk between these pathways, forming a complicated and robust cellular signal transduction network that is regulated by compensatory mechanisms. Therefore, inhibitors that target only a single pathway might not be effective initially and their long-term use might generate drug resistance (18). These problems highlight the need for the identification of novel cancer chemotherapies. Natural products, such as traditional Chinese medicines (TCMs), have been used clinically to treat various types of diseases including cancer (19). Their effect, in general, is believed to be the result of targeting multiple molecular pathways. Moreover, TCMs have relatively fewer side effects as compared to modern chemotherapeutics. Therefore, TCMs have gained recent attention as potential chemotherapeutics for CRC.

Many traditional Chinese medicinal herbs, such as *Hedyotis diffusa*, *Spica prunella* and *Scutellaria barbata*, have long been used in China for the clinical treatment of CRC (20-23). Ursolic acid (UA), a pentacyclic triterpene acid, is a biologically active compound present in these herbs. Previous studies report that UA exhibits a broad range of pharmacological properties such as anti-inflammatory, antiviral, antioxidant, hepatoprotective, cytotoxic, antitumor, anti-angiogenesis and anti-metastatic activities (24). Recent studies have shown that UA inhibits the proliferation and induces apoptosis and inhibits proliferation of colon carcinoma cells (25,26). In this study, we further evaluated the efficacy of UA as a therapeutic agent for CRC in vivo and in vitro and investigated the underlying molecular mechanisms of its action.

Materials and methods

**Materials and reagents.** Ursolic acid (UA) was purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Matrigel was provided by Becton-Dickinson (San Jose, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and TRIZol reagent were purchased from Invitrogen (Carlsbad, CA, USA). TUNEL assay kit (TumorTACS *in situ*) was purchased from R&D Systems (Minneapolis, MN, USA). BCA Protein Assay kit was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). All antibodies were purchased from Cell Signaling Technology (CST, Beverly, MA, USA). Bio-Plex phosphoprotein assay kits were purchased from Bio-Rad (Hercules, CA, USA). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich.

**Cell culture.** Human colon carcinoma HT-29 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HT-29 cells were grown in DMEM. DMEM was supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were cultured at 37°C, 5% CO₂ and in a humidified environment.

**Animals.** Male BALB/c athymic (nude) mice (with an initial body weight of 20-22 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed under pathogen-free conditions with controlled temperature (22°C), humidity and a 12-h light/dark cycle. Food and water were given *ad libitum* throughout the experiment. All animal treatments were performed strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

**In vivo nude mice xenograft study.** CRC xenograft mice were produced with HT-29 cells as described previously (21). At 5 days following xenograft implantation (tumor size ~3 mm in diameter), mice were randomized into two groups (n=10) and treated with UA (dissolved in PBS) 12.5 mg/kg or saline by daily intraperitoneal injection, 6 days a week for 16 days. Body weight and tumor size were measured. Tumor size was determined by measuring the major (L) and minor (W) diameter with a caliper. The tumor volume was calculated according to the following formula: tumor volume = π/6 x L x W². At the end of the experiment, the animals were anaesthetized with pelltobarbitalum natrium and tumors were excised and weighed.

**Cell viability evaluation by MTT assay.** UA was dissolved in DMSO and diluted to working concentrations with culture medium. The final concentration of DMSO in the medium for all cell-based experiments was 0.1%. HT-29 cells were seeded into 96-well plates at a density of 1.0x10⁴ cells/well in 0.1 ml medium. The cells were treated with various concentrations of UA for 24 h or with 40 µM of UA for different periods of time. Treatment with 0.1% DMSO was included as the vehicle control. At the end of the treatment, 10 µl MTT [5 mg/ml in phosphate-buffered saline (PBS)] were added to each well and the samples were incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 µl DMSO. Absorbance was measured at 570 nm using an ELISA reader (BioTek, Model EXL800, USA).

**Colony formation assay.** HT-29 cells were seeded into 6-well plates at a density of 2x10³ cells/well and treated with various concentrations of UA for 24 h. The cells were harvested, resuspended in medium in the absence of UA and reseeded into 6-well plates at a density of 1.5x10³ cells/well. After incubation for 7 days in a 37°C humidified incubator with 5% CO₂, colonies were counted under light microscopy. Cell survival was calculated by normalization to the survival of control (untreated) cells.

**Cell cycle analysis.** Cell cycle analysis was carried out by flow cytometry using FACS analysis with propidium iodide (PI) staining. HT-29 cells were treated with various concentrations of UA for 24 h, harvested, adjusted to a concentration...
of 1x10^6 cells/ml and fixed in 70% ethanol at 4°C overnight. The fixed cells were washed twice with cold PBS and incubated for 30 min with RNase (8 µg/ml) and PI (10 µg/ml). The fluorescent signal was detected through the FL2 channel and the proportion of DNA in different phases was analyzed using ModFitLT Version 3.0 (Verity Software House, Topsham).

**Apoptosis detection in CRC tumor tissues by TUNEL staining.** Formalin-fixed (12 h) and paraffin-embedded sections of tumors (4-µm-thick) were analyzed by TUNEL staining using TumorTACS in situ kit (R&D Systems). Apoptotic cells were counted as DAB-positive cells (brown stained) at five arbitrarily selected microscopic fields at a magnification of x400. TUNEL-positive cells were counted as a percentage of the total cells.

**Apoptosis detection in HT-29 cells by flow cytometry analysis with Annexin V/PI staining.** After incubation with various concentrations of UA, apoptosis of HT-29 cells were determined by flow cytometry using a fluorescence-activated cell sorting (FACS) caliber (Becton-Dickinson) and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Becton-Dickinson). Staining was performed according to the manufacturer's instructions. The percentage of cells in early apoptosis was calculated by Annexin V-positivity and PI-negativity and the percentage of cells in late apoptosis was calculated by Annexin V-positivity and PI-positivity.

**DNA fragmentation analysis in HT-29 cells by gel electrophoresis.** DNA fragmentation was determined using a DNA ladder detection kit (Invitrogen), following the manufacturer's instructions. Briefly, HT-29 cells were detached from the culture plates by scraping and washed in PBS. DNA was isolated from 2x10^6 cells. The DNA was analyzed after separation by gel electrophoresis. The DNA bands were examined using a gel documentation system (Model Gel Doc 2000, Bio-Rad).

**Immunohistochemical analysis of CRC tumor tissues.** Six tumors were randomly selected from UA-treatment or control groups. Tumor tissues were fixed in 10% formaldehyde for 12 h, paraffin-embedded, sectioned and placed on slides. The immunohistochemical analysis was carried out as described previously (21). Briefly, the slides were subjected to antigen retrieval and endogenous peroxidase activity was quenched using the true color multi-functional cell image analysis management system (Image-Pro Plus, Media Cybernetics, USA). To control for non-specific staining, PBS was used to replace the primary antibody as a negative control.

**RT-PCR analysis.** Total RNA was isolated from tumor tissues (three tumors were randomly selected from UA-treatment or control groups) or HT-29 cells with TRizol reagent. Oligo(dT)-primed RNA (1 µg, isolated from tumor tissues or cells) was reverse-transcribed with SuperScript II reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The obtained cDNA was used to determine the level of Bcl-2, Bax, Cyclin D1, CDK4 and p21 mRNA by PCR with Taq DNA polymerase (Fermentas). GAPDH was used as an internal control. Samples were analyzed by gel electrophoresis (1.5% agarose). The DNA bands were examined using a gel documentation system (Model Gel Doc 2000, Bio-Rad).

**Western blot analysis.** The western blot analysis was carried out as described previously (21). Briefly, tumors and HT-29 cells were separately lysed in mammalian cell lysis buffer with different protein inhibitor. Equal amounts of protein from each tumor or cell lystate was subjected to SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked for 2 h with 5% non-fat dry milk and incubated with the desired primary antibody directed against p-STAT3, total-STAT3, Bcl-2, Bax, Cyclin D1, CDK4, p21, or β-actin (all in 1:1,000 dilutions) overnight at 4°C. Appropriate HRP-conjugated secondary antibodies with chemiluminescence detection were used to image the antibody-detected proteins.

**Bio-Plex phosphoprotein assay.** Eight tumors were randomly selected from UA-treatment or control groups and homogenized. HT-29 cells (2.5x10^6) were seeded into 25 cm^2 flasks in 5 ml medium and treated with 40 µM of UA for 24 h. Tumor tissues and treated cells were lysed using a commercially available lysis kit (Bio-Rad Laboratories) and centrifuged at 14,000 x g for 15 min. The protein extracts were quantified by BCA protein assay. The presence of p-ERK1/2, p-JNK and p-p38 was detected using a bead-based multiplex assay for phosphoproteins (Bio-Plex Phosphoprotein assay, Bio-Rad Laboratories) according to the manufacturer's protocol. Data was collected and analyzed using the Bio-Plex 200 suspension array system (Bio-Rad).

**Statistical analysis.** Data were presented as mean ± SD for the indicated number of independently performed experiments. The data were analyzed using the SPSS package for Windows (Version 17.0). Statistical analysis was carried out with Student's t-test and ANOVA. Differences with P<0.05 were considered to be statistically significant.

**Results**

**UA inhibits tumor growth in colorectal cancer (CRC) xenograft mice.** The in vivo antitumor effect of UA was evaluated through the same mice by measuring changes in body weight. As shown in Fig. 1A, UA treatment resulted in 55.9% less tumor volume as compared to control (control, 0.93±0.21 cm^3; UA-treatment, 0.41±0.13 cm^3, P<0.01). In a consistent manner, the tumor weights in the UA-treatment group was 65.7% less than in the control group (control, 0.67±0.15 g; UA-treatment, 0.23±0.11 g, P<0.01, Fig. 1B). However, administration of UA had no effect on gain in body weight during the course of the study (Fig. 1C), suggesting that UA is potent in suppressing colorectal tumor growth in vivo, without noticeable toxicity.
UA inhibits proliferation of colorectal cancer cells via G1/S cell cycle arrest and modulation of Cyclin D1, CDK4 and p21 expression. The in vivo effect of UA on CRC cell proliferation was determined via immunohistochemical staining (IHS) for PCNA, a proliferation marker that is specifically expressed in proliferating cell nuclei. As shown in Fig. 2A,
the percentage of PCNA-positive cells in tumor tissues from control and UA-treated CRC xenograft mice was 41.0±5.9% and 25.0±3.4%, respectively (P<0.01), suggesting that UA represses cell proliferation in tumor tissues.

We next sought to examine the effect of UA on CRC cell proliferation in vitro. We treated human colon carcinoma HT-29 cells with various concentrations of UA and measured the amount of surviving cells with the MTT assay. As shown in Fig. 2B, treatment with 20-120 µM of UA for 24 h dose-dependently reduced the viability of HT-29 cells (P<0.01). The estimated half maximal inhibitory concentration (IC$_{50}$) of UA in HT-29 cells was ~35 µM. We next evaluated the effect of UA on cell viability as a function of time. We found that treatment with UA (40 µM, approximately the IC$_{50}$) led to a gradual decrease in cell viability with an increase in exposure time (P<0.01, Fig. 2C). We further verified these results using a colony formation assay. UA treatment dose-dependently reduced the cell survival rate (P<0.01, Fig. 2D). Thus, UA inhibits CRC cell proliferation in vitro dose- and time-dependently.

G1/S transition is one of the two main cell cycle checkpoints that are critical for regulation of cell proliferation, which is strongly regulated by Cyclin D1, CDK4 and p21. We therefore investigated the effect of UA on the G1 to S progression and on the expression of these regulatory genes. Using PI staining followed by FACS analysis we found that the percentage HT-29 cells in S-phase following treatment with UA was decreased in a dose-dependent manner (Fig. 2E, P<0.01). In addition, data from RT-PCR and western blot analysis showed that UA treatment significantly reduced the mRNA and protein levels of pro-proliferative Cyclin D1 and CKD4 both in CRC tumors and HT-29 cells, whereas the mRNA and protein levels of anti-proliferative p21 was significantly increased after UA treatment (Fig. 3, P<0.01). These data together suggest that UA inhibits CRC cell proliferation through blockade of G1-S progression and the modulation of the expression of cell cycle-regulatory genes.

UA induces colorectal cancer cell apoptosis via alteration in the Bax/Bcl-2 ratio. To further evaluate the effect of UA on CRC cell growth, we examined whether UA induces apoptosis. Apoptosis in tumors was evaluated via TUNEL. As shown in Fig. 4A, the percentage of TUNEL-positive cells was greater in tumors from UA-treated mice as compared to controls (UA-treatment, 36.7±8.6%; control, 26.3±2.0%; P<0.01). UA-induced apoptosis in vitro was examined using HT-29 cells and Annexin V/PI staining followed by FACS analysis. As shown in Fig. 4B and C, UA treatment increased the percent of cells undergoing apoptosis [early apoptosis found within the lower right (LR) and late apoptosis upper right (UR) quadrants in the FACS diagram] in a dose-dependent fashion (P<0.05). The in vitro pro-apoptotic activity of UA was confirmed by its effect on cellular DNA fragmentation, a typical feature of apoptosis. As shown in Fig. 4D, DNA extracted from HT-29 cells treated with UA displayed a characteristic ladder pattern of discontinuous DNA fragments.

To investigate the mechanism of the pro-apoptotic activity of UA, we examined its effect on the expression of Bcl-2 family proteins that are important regulators of apoptosis. As shown in Fig. 5A and B, UA significantly reduced anti-apoptotic
Bcl-2 mRNA level both in the tumors of CRC mice and HT-29 cells, whereas the level of pro-apoptotic Bax mRNA was significantly increased after UA treatment. The protein expression patterns of Bcl-2 and Bax were similar to the patterns observed for the respective mRNA (Fig. 5C and D). Taken together, these data demonstrate that UA promotes colorectal cancer cell apoptosis both \textit{in vivo} and \textit{in vitro} through an increase in the pro-apoptotic Bax/Bcl-2 ratio.

UA suppresses multiple signaling pathways in CRC cells. To further explore the underlying mechanisms of the observed anticancer activities of UA, we determined its effect on the activation of several CRC-related signal transduction cascades including STAT3, ERK, JNK and p38. Activation of STAT3 is mediated by its phosphorylation at tyrosine 705, we therefore investigated the effect of UA on STAT3 activation in CRC by western blot analysis using an antibody that recognizes STAT3 phosphorylation at Tyr\textsuperscript{705}. Since many cultured human cancer cell lines including HT-29 do not express constitutively phosphorylated STAT3 \textit{in vitro}, we stimulated STAT3 activation in HT-29 cells with 10 ng/ml of IL-6 for 15 min. As shown in Fig. 6A, UA profoundly inhibited IL-6 induced phosphorylation of STAT3 in HT-29 cells. In a consistent fashion, UA treatment significantly decreased the level of phosphorylated STAT3 in tumors of CRC xenograft mice (Fig. 6B). The levels of non-phosphorylated STAT3 in tumor HT-29 cells and tumor tissues remained unchanged with UA treatment.

The activation (phosphorylation) of ERK1/2, JNK and p38 in CRC xenograft tumor tissues and HT-29 cells was determined by Bio-Plex Phospho-protein assay. As shown in Fig. 6C and D, after UA treatment the phosphorylation levels of ERK1/2, JNK and p38 in both tumors and HT-29 cells were decreased as compared to controls (P<0.01). Collectively, these data suggest that UA significantly suppresses the activation of multiple CRC-related signaling pathways.
Discussion

Cancer development is strongly correlated with the aberrant activation of multiple intracellular signal transduction pathways which usually function redundantly. In addition, crosstalk between these pathways generates a complicated and robust signaling network that is regulated by compensatory mechanisms. Given the complexity of cancer pathogenesis, ...
and progression, many of the currently used antitumor agents, which typically target a single intracellular pathway, might not always be effective on complex tumor systems. In contrast, long-term use of these single-target-based agents often generates drug resistance. Moreover, most currently used chemotherapies contain intrinsic toxicity against normal cells. Therefore, the development of novel anticancer chemotherapies is urgently needed. Natural products have received great interest since they have relatively fewer side effects as compared to modern chemotherapeutics and have been shown to display multiple therapeutic effects for various diseases including cancer. Ursolic acid (UA), a major active compound of many traditional Chinese medicinal herbs, has been shown to possess anticancer activity. However, the precise mechanism of its potential tumoricidal activity remains largely unclear. Therefore, before UA can be further developed as an anticancer agent, the mode of action for its antitumor effects should be fully elucidated.

Cancer cells are characterized by an uncontrolled increase in cell proliferation and/or a reduction in cell apoptosis. The mitochondrion-dependent pathway is the most common apoptotic pathway in vertebrate animal cells, which is highly regulated by Bcl-2 family proteins including anti-apoptotic members such as Bcl-2 and pro-apoptotic members such as Bax (27). The ratio of active anti- and pro-apoptotic Bcl-2 family members determines the fate of cells and alteration of the ratio by aberrant expression of these proteins impairs the normal apoptotic program contributing to various apoptosis-related diseases including cancer (27,28). Higher Bcl-2-to-Bax ratios due to the overexpression of Bcl-2 or downregulation of Bax expression are commonly found in cancer, which not only confers a survival advantage to the cancer cells but also causes resistance to chemo- and radio-therapies. Eukaryotic cell proliferation is primarily regulated by the cell cycle. G1/S transition is one of the two main checkpoints of the cell cycle and is responsible for initiation and completion of DNA replication (29). G1/S progression is strongly regulated by Cyclin D1 that exerts its function via forming an active complex with its CDK major catalytic partners (CDK4/6) (30). An unchecked or hyperactivated Cyclin D1/CDK4 complex often leads to uncontrolled cell division and malignancy (31,32). As a proliferation inhibitor, p21 protein plays a role in G1 arrest by binding to and inhibiting the activity of Cyclin-CDK complexes; and the decrease of p21 is associated with the promotion of tumor formation and a poor prognosis in many types of cancer (33). Therefore, re-balancing of cell apoptosis and proliferation via regulation of the expression of apoptosis- or cell cycle-related genes is a promising target for anticancer therapies. Using a CRC mouse xenograft model, we demonstrated that UA significantly suppress the activation of these CRC-related signaling pathways both in vivo and in vitro.

In conclusion, we report for the first time that UA possesses a broad range of anticancer activities due to its ability to affect multiple intracellular targets. Our findings suggest that UA could be a novel therapeutic agent for the treatment of colorectal and possibly other cancers.

Acknowledgements

This study was sponsored by the National Natural Science Foundation of China (no. 81073097), the Developmental Fund of Chen Keji Integrative Medicine (no. CKJ 2011001), and the China Postdoctoral Science Foundation (no. 2012M511437).

References