Cancer Therapy: Preclinical

Ursolic Acid Inhibits Growth and Metastasis of Human Colorectal Cancer in an Orthotopic Nude Mouse Model by Targeting Multiple Cell Signaling Pathways: Chemosensitization with Capecitabine

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Abstract

Purpose: Development of chemoresistance, poor prognosis, and metastasis often renders the current treatments for colorectal cancer (CRC) ineffective. Whether ursolic acid, a component of numerous medicinal plants, either alone or in combination with capecitabine, can inhibit the growth and metastasis of human CRC was investigated.

Experimental design: The effect of ursolic acid on proliferation of CRC cell lines was examined by mitochondrial dye uptake assay, apoptosis by esterase staining, NF-κB activation by DNA-binding assay, and protein expression by Western blot. The effect of ursolic acid on the growth and chemosensitization was also examined in orthotopically implanted CRC in nude mice.

Results: We found that ursolic acid inhibited the proliferation of different colon cancer cell lines. This is correlated with inhibition of constitutive NF-κB activation and downregulation of cell survival (Bcl-xl, Bcl-2, cFLIP, and survivin), proliferative (cyclin D1), and metastatic (MMP-9, VEGF, and ICAM-1) proteins. When examined in an orthotopic nude mouse model, ursolic acid significantly inhibited tumor volume, ascites formation, and distant organ metastasis, and this effect was enhanced with capecitabine. Immunohistochemistry of tumor tissue indicated that ursolic acid downregulated biomarkers of proliferation (Ki-67) and microvessel density (CD31). This effect was accompanied by suppression of NF-κB, STAT3, and β-catenin. In addition, ursolic acid suppressed EGF receptor (EGFR) and induced p53 and p21 expression. We also observed bioavailability of ursolic acid in the serum and tissue of animals.

Conclusion: Overall, our results show that ursolic acid can inhibit the growth and metastasis of CRC and further enhance the therapeutic effects of capecitabine through the suppression of multiple biomarkers linked to inflammation, proliferation, invasion, angiogenesis, and metastasis. Clin Cancer Res; 18(18); 4942–53. ©2012 AACR.

Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death among men ages 40 to 79 years (1), with an annual global mortality of greater than 639,000 persons. In the year 2012 in the United States alone, 73,420 men and 70,480 women will be diagnosed with CRC. Deaths from CRC are expected to be 51,690 in 2012, up from 49,920 in 2009. Chemotherapy, radiotherapy, and surgery are the basic treatment methods used against CRC. However, long-term use of chemotherapy can make patients feel very sick and can also make their condition worse. Tumors also develop resistance to the chemotherapy over time.

Accumulating evidence suggests that the development and progression of many malignancies, including CRC, are associated with constitutive activation of multiple signaling pathways that promote proliferation, inhibit apoptosis, and induce metastasis (2). NF-κB and STAT3 transcription factors, which primarily regulate inflammation, play a crucial role in regulating several pathways that affect tumor cell survival, angiogenesis, motility, and invasiveness (3). EGF receptor (EGFR), one of the family of 4 erbB receptors, is found to be overexpressed in a variety of human tumors, including CRCs (4). Aberrant activation of EGFR and the
Ursolic Acid Inhibits Growth and Metastasis of CRC

Translational Relevance

Despite advances in earlier detection and therapy, colorectal cancer (CRC) is the leading cause of death from gastrointestinal malignancy. Existing chemotherapeutic regimens are associated with lack of efficacy and yet are highly toxic. Long-term use of chemotherapeutic drugs also resulted into the development of tumor resistance over time. Thus the agents, which can overcome the resistance and can enhance the effect of chemotherapeutic drugs, are urgently needed. Through in vitro and in vivo experiments, we show for the first time that ursolic acid, an active compound present in numerous medicinal plants including holy basil, can potentiate the effect of capecitabine, a standard drug, against CRC. Our preclinical findings show that ursolic acid is effective in inhibition of tumor growth and metastasis and that this effect is further enhanced when combined with capecitabine. This antitumor effect of ursolic acid was mediated through multiple pathways linked to inflammation, proliferation, invasion, angiogenesis, and metastasis.

EGF signal pathway is associated with neoplastic cell proliferation, migration, stromal invasion, resistance to apoptosis, and angiogenesis (5). Thus, agents that can control these multiple signaling pathway have potential for use against CRC.

Although natural remedies have been claimed to have potential for the prevention or treatment of CRC, neither an active component nor a mechanism has been well characterized. Ursolic acid, a pentacyclic triterpenoid, as a common active compound of numerous medicinal plants including holy basil (6) has been shown to exhibit anticancer potential in vitro and in vivo. It suppressed proliferation and induced apoptosis in cells of various cancers, including breast cancer (7), colon cancer (8), non-small cell lung cancer (9), cervical cancer (10), multiple myeloma (11), pancreatic cancer (12), melanoma (13), and prostate cancer (14). In animal models, ursolic acid inhibited tumorigenesis (15–17), suppressed tumor invasion (18), and inhibited metastasis of esophageal carcinoma (19).

How ursolic acid acts as an anticancer agent is not yet clear. However, several reports have suggested that it inhibits DNA replication (20), induces Ca^{2+} release (21), activates caspases (13, 22) and c-jun-NH2-kinase (JNK; ref. 14), downregulates antiapoptotic genes (23, 24), inhibits COX-2 and iNOS (25, 26), suppresses MMP-9 (18), and inhibits protein tyrosine kinase (27), STAT3 (11), and NF-kB activation (24). All these reports suggest that ursolic acid has potential against CRC.

In the current report, we investigated whether ursolic acid inhibits the growth and metastasis of human colon cancer in vitro and in an orthotopic mouse model of CRC. We found that ursolic acid significantly inhibited the growth and metastasis of CRC and further enhanced the effect of capecitabine through downregulation of multiple cell signaling pathways.

Materials and Methods

Materials

Ursolic acid (Fig. 1A) was kindly supplied by King Sing Guan (Haikou, China), and its purity was ascertained by high-performance liquid chromatography (HPLC; Fig 1A, left). Polyclonal antibodies against p65, ICAM-1, cyclin D1, MMP-9, survivin, and c-IAP-1, and monoclonal antibodies against VEGF, c-myc, Bcl-2, and Bcl-xl were obtained from Santa Cruz Biotechnology. The liquid 3,3-diaminobenzidine + substrate chromogen system–horseradish peroxidase used for immunohistochemistry was obtained from DakoCytomation. Penicillin, streptomycin, Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium, and FBS were obtained from Invitrogen. All other chemicals were obtained from Sigma unless otherwise stated.

Cell lines

The human colon cancer cell lines, HCT116, HT29, and Caco2, were obtained from American Type Culture Collection. HCT116 cells were cultured in DMEM, and HT29 and Caco2 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Proliferation assay

The effect of ursolic acid on cell proliferation was determined by the MTT uptake method as described previously (28). The absorbance was measured at 570 nm using an MRX Revelation 96-well multisScanner (Dynex Technologies).

Clonogenic assay

HCT116 cells (3,000 cells/well) were seeded in 12-well plates, incubated for 12 hours, and treated with different concentrations of ursolic acid for 12 hours. The cells were then reseeded in 6-well plates and allowed to form colonies for 14 days. Colonies were stained with 0.3% crystal violet solution for 20 minutes. The excess crystal violet solution was washed with distilled water to visualize the clonogenic potential of the cells.

Apoptosis assay

To determine whether ursolic acid can potentiate the apoptotic effects of capecitabine in colon cancer cells, we used a Live/Dead Assay Kit (Molecular Probes). In brief, cells (5,000 cells per well) were incubated in chamber slides and coincubated with ursolic acid and capecitabine for 24 hours. Cells were then stained with assay reagents for 30 minutes at ambient temperature. Cell viability was determined by a fluorescence microscope by counting live (green) and dead (red) cells.

Animals

Male athymic nu/nu mice (4 weeks old) were obtained from the breeding section of the Department of...
Experimental Radiation Oncology at The University of Texas MD Anderson Cancer Center (Houston, Texas). Three mice per cage were housed in standard acrylic glass mouse cages in a room maintained at constant temperature and humidity with a 12-hour light:dark cycle; mice were fed regular sterilized chow diet with water ad libitum. Our experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the MD Anderson Cancer Center.

Orthotopic implantation of HCT116 cells

HCT116 cells were stably transfected with luciferase as previously described (28). Luciferase-transfected HCT116 cells were harvested from subconfluent cultures after a brief exposure to 0.25% trypsin and 0.2% EDTA. Trypsinization was stopped with medium containing 10% FBS. The cells were washed once in serum-free medium and resuspended in PBS. Only suspensions consisting of single cells, with more than 90% viability, were used for the injections. Mice were anesthetized with ketamine-xylazine solution, a small, left abdominal flank incision was made, and HCT116 cells (1.5 \times 10^6 cells) in 50 μL of PBS were injected into the cecum using a 30-gauge needle and a calibrated push button-controlled dispensing device (Hamilton Syringe Company). To prevent leakage, a cotton swab was held cautiously for 1 minute over the site of injection. The abdominal wound was closed in one layer with wound clips (Braintree Scientific, Inc).

Experimental protocol

One week after implantation, mice were randomly assigned to the following treatment groups (6 mice per group): (i) corn oil (vehicle; 100 μL daily); (ii) ursolic acid alone (250 mg/kg, once daily, orally); (iii) capecitabine alone (250 mg/kg, twice weekly by gavage), and (iv) ursolic acid (250 mg/kg, once daily, orally), and capecitabine.
(60 mg/kg, twice weekly by gavage). In combination group, ursolic acid was given 6 to 8 hours before capecitabine. Tumor volumes were monitored weekly by the bioluminescence imaging system (IVIS) 200 and Living Image software (Caliper Life Sciences). Before imaging, animals were anesthetized in an acrylic chamber with 2.5% isoflurane/air mixture and injected intraperitoneally with o-luciferin potassium salt in PBS at a dose of 150 mg/kg body weight. After 10 minutes of incubation with luciferin, mice were placed in a right later decubitus position, and a digital gray scale animal image was acquired, followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the animal. Signal intensity was quantified as the sum of all detected photons within the region of interest per second per steradian. Mice were imaged on days 0, 7, 14, 21, and 28 of treatment. Therapy was continued for 4 weeks, and the mice were sacrificed 1 week later. Primary tumors in the cecum were excised, and the final tumor volume was measured as $V = \frac{2}{3} \pi r^3$, where $r$ is the mean of the 3 dimensions (length, width, and depth). The number of metastasis colonies was counted in the liver, intestines, lungs, rectum, and spleen. Each tumor excised from mice was divided into 3 parts. The first part of the tumor tissue was formalin-fixed, second part fixed in optimum cutting temperature (OCT) and third part was snap frozen in liquid nitrogen and stored at −80°C. Hematoxylin and eosin staining confirmed the presence of tumors in each cecum.

**NF-κB activation in colon cancer cells**

To assess NF-κB activation, we isolated nuclei from the colon cancer cell lines and conducted the electrophoretic mobility shift assay (EMSA) essentially as previously described (29).

**Immunolocalization of NF-κB p65, β-catenin, VEGF, and MMP-9 in tumor samples**

The nuclear localization of p65 and the expressions of MMP-9 and VEGF were examined using an immunohistochemical method described previously (28). Pictures were captured with a Photometrics CoolSNAP CF color camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging).

**Ki-67 immunohistochemistry**

Frozen sections (5 μm) were stained with anti-Ki-67 (rabbit monoclonal clone SP6; NeoMarkers) antibody as previously described (28). Results for Ki-67–positive cells are shown as ×40 magnification. A total of ten ×40 fields were examined from 3 tumors of each of the treatment groups.

**Microvessel density**

Frozen sections (5 μm) were fixed in cold acetone and stained with rat anti-mouse CD31 monoclonal antibody (Pharmingen) as previously described (28). The CD31-stained slides were observed under Leica DM4000B fluorescence microscope (Leica Microsystems, Inc.) equipped with a SPOT-RTKE digital camera, and the images were acquired and stored using SPOT advanced software (Diagnostic Instruments).

**Protein extraction and Western blot analysis**

Colorectal tumor tissues (75–100 mg) from control and experimental mice were minced and incubated on ice for 30 minutes in 0.5 mL of ice-cold whole-cell lysis buffer. The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 16,000 × g at 4°C for 10 minutes. The proteins were then fractionated by SDS-PAGE, electro-transferred to nitrocellulose membranes, blotted with each antibody, and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**HPLC analysis**

The extraction of ursolic acid was conducted as described by Chen and colleagues (30). Frozen tissue (100 mg) was thawed, 400 μL of ethanol was added, and the mixture was homogenized for 30 seconds. Next, 500 μL of water was added, and the solution was homogenized for 15 seconds. Then, 500 μL of hexane was added, and the solution was again homogenized for 15 seconds. The samples were centrifuged at 5,000 rpm for 5 minutes at 4°C. The organic layer was evaporated to dryness under purified air at 40°C. The pellets were reconstituted with mobile phase and estimated by HPLC (Waters Inc.). A silica-based C18 column and a mobile phase that was the mixture of acetoni-trile: methanol:acetic acid (10%) in a ratio of 45:45:10 were used for separation. Analyses were conducted at a flow rate of 0.4 mL/min with the detector operating at a wavelength of 291 nm. Tissues from vehicle-treated mice were set as controls that reflect the baseline.

**Combination effects of ursolic acid and capecitabine**

Assessment of synergistic drug combination treatments between ursolic acid and capecitabine were evaluated using MTT assays on HCT116 cells. A total of 4.0 × 10^4 cells were plated in triplets and treated with ursolic acid alone (0.25 × IC50, 0.5 × IC50, 1 × IC50, 2 × IC50, 4 × IC50), capecitabine alone (0.25 × IC50, 0.5 × IC50, 1 × IC50, 2 × IC50, 4 × IC50), and ursolic acid in combination with capecitabine at fixed ratio (Supplementary Table S1). After incubation, 5.0 mg/mL MTT reagent was added into each well, incubated for 2 hours in the dark at 37°C and then MTT lysis buffer was added as described previously (28). The absorbance was measured at 570 nm using an MRX Revelation 96-well multiscanner (Dynex Technologies).

**Statistical analysis**

For the effect of ursolic acid on cell proliferation over time, we modeled the percentage of viable cells using linear mixed effects models with fixed effects as dose, day of measurement, and their interaction and (replicated) cell growth as random effects. In cases where the fixed effects were statistically significant, pairwise comparisons between doses (within each time point) were conducted by
comparing the least square means. Multiple comparison adjustments were made using the Bonferroni method. Similarly, we examined the effect of ursolic acid and capecitabine on tumor growth of nude mice over time using a linear mixed model with fixed effects as ursolic acid or capecitabine, day of measurement, and interactions between treatment and day (other 2-way and 3-way interactions were not significant), along with random effect of mice nested within treatment group. Repeated measurements over time were modeled using an (first order) auto-regressive covariance structure.

To model the effects of ursolic acid and capecitabine on apoptosis, metastatic score, and tumor weight of nude mice, we first modeled the outcomes using linear models with fixed effects as ursolic acid and capecitabine, and their interaction. Pairwise comparisons were conducted as above with multiple comparison adjustments made using the Bonferroni’s method. When multiple cell lines were tested, we examined each cell line separately.

For all linear models, we examined the normality assumption of the residuals using Q-Q plots and used transformation to approximate normality where this assumption was violated (e.g. for tumor weight we used square-root transformation). We computed the incidence rate of ascites with exact 95% confidence interval. The differences in ascites incidence rate among treatment groups were compared using Fisher exact test.

Assessment of the type of drug interactions was done using Chou and Talalay method. Assuming that a dose-effect curve follows Chou and Talalay median effect equation, \( E = \frac{(d/C_0)^m}{1+(d/C_0)^m} \), where \( d \) is the dose of a drug, \( D_m \) is the median effective dose of the drug, and \( m \) is a slope parameter. The above equation can be rewritten as \( \log \frac{1}{E} = m \log d - \log D_m \). We regress \( \log \frac{1}{E} \) on \( \log d \) to get a marginal dose-effect curve. Then the dose level to achieve certain effect can be estimated for each of the 3 regimens (drug 1 alone, drug 2 alone, and the combination of drugs 1 and 2 with constant relative potency between them). Suppose that combination dose \( (d_1, d_2) \) elicits the same effect \( y \) as drug 1 alone at dose \( D_{y,1} \), and drug 2 alone at dose \( D_{y,2} \), then the interaction index \( II \) as defined by the Loewe additivity model (31), \( II = \frac{D_{y,1}}{D_{y,1} + D_{y,2}} \), which measures the magnitude of drug interaction. The value of \( II \) is estimated by the above equation and the corresponding 95% confidence interval is computed using the delta method (32) to calculate the variance of \( II \). The value of \( II < 1 \), \( II = 1 \), and \( II > 1 \) correspond to the drug interactions being synergistic, additive, and antagonistic, respectively. Statistical software SAS 9.1.3 (SAS) and S-Plus 8.0 (TIBCO Software Inc.) were used for all the analyses.

Results

Our goal in the current study was to determine whether ursolic acid can affect the growth and metastasis of CRC and enhance the effect of capecitabine, which is used routinely to treat CRC, and if so, to determine the mechanism by which ursolic acid manifests its effects in vitro and in vivo against human CRC. We used 3 different well-characterized human colon cancer cell lines (HCT116, HT29, and Caco2) that exhibit distinct characteristics. To facilitate the monitoring of tumor growth in animals, HCT116 was stably transfected with the luciferase reporter gene and used in an orthotopic transplant model in mice.

Ursolic acid inhibits the proliferation of CRC cells in vitro

We first determined whether ursolic acid inhibits the proliferation of human CRC cells. We used one cell line with mutant K-ras (HCT116) and 2 cell lines with wild-type K-ras (HT29 and Caco2). We found that ursolic acid significantly \((P < 0.001)\) inhibited cell proliferation in all 3 cell lines in a dose- and time-dependent manner. HT29 and Caco2 cells were found to be more sensitive than HCT116 cells as indicated by their IC\(_{50}\) value (HT29, 8.7 \(\mu\)mol/L; Caco2, 6.2 \(\mu\)mol/L, and HCT116, 9.8 \(\mu\)mol/L; Fig. 1A, right).

Ursolic acid alone and in combination with capecitabine downregulates the expression of proteins linked to survival, proliferation, and metastasis in CRC cells

We also examined whether ursolic acid alone or in combination with capecitabine can downregulate the expression of proteins associated with survival, proliferation, invasion, and metastasis. Our results showed that ursolic acid inhibited the expression of CRC survival (c-FLIP, Bcl-2, survivin, Bcl-xL), proliferation (cyclin D1), and metastasis (MMP-9, VEGF, ICAM-1) proteins in dose-dependent manner (Fig. 1B, left). When ursolic acid was treated in combination with capecitabine, the effect was prominent (Fig. 1B, right). However, induction of cFLIP, Bcl-2, VEGF, and ICAM-1 was observed by capecitabine alone treatment.

Ursolic acid inhibits the constitutive action of NF-\(\kappa\)B in CRC cells

Because NF-\(\kappa\)B activation has been closely associated with proliferation of tumor cells (33) and CRC is known to express constitutively active NF-\(\kappa\)B, we investigated whether ursolic acid inhibits this transcription factor in colon cancer cells. Therefore, we conducted the DNA-binding assay to determine whether ursolic acid inhibits constitutive NF-\(\kappa\)B activation. We found that ursolic acid inhibited NF-\(\kappa\)B activation in a dose-dependent manner (Fig. 1C).

Ursolic acid suppresses colony formation

Next, we determined whether ursolic acid could affect the long-term colony formation assay. We found that ursolic acid treatment significantly suppressed the colony-forming ability of HCT116 tumor cells in a dose-dependent manner (Fig. 1D, left).
Ursolic acid sensitizes CRC cells to apoptosis by capecitabine

We next investigated whether ursolic acid can enhance the effect of capecitabine-induced apoptosis in all 3 cell lines. For this, we conducted the Live/Dead apoptosis assay. Our linear models showed that there was significant interaction between the 2 drugs for all 3 cell lines. The ursolic acid significantly enhanced capecitabine-induced apoptosis on all 3 cell lines (P value for interaction term, <0.001; Fig. 1D, right). These observations together suggested that ursolic acid has potential in the treatment of CRC. Therefore we designed in vivo studies.

Ursolic acid synergistically enhances capecitabine-induced cytotoxicity

On the basis of MTT assay and Chou and Talalay median effect results, it was found that ursolic acid was able to reduce the viability of HCT116 cells to a higher extent when used in combination with capecitabine. Drug interaction index (II) analysis indicates synergistic effects for all combinations where ursolic acid and capecitabine were used equal to or higher than IC50 concentration. However, additive effects were observed where ursolic acid and capecitabine were used less than IC50 concentration (Supplementary Table S1).

Ursolic acid inhibits the growth of human CRC in an orthotopic nude mouse model

On the basis of these results, we decided to determine whether ursolic acid affects the growth of CRC, alone and in combination with capecitabine. For this, we used orthotopically implanted human HCT116 cancer cells in nude mice. The experimental protocol is depicted in Fig. 2A. Luciferase-transfected HCT116 cells were implanted in the
After 1 week, on the basis of the initial IVIS image, we randomly assigned the mice to 4 groups (6 mice per group) and began the treatment as per the experimental protocol. The treatment was continued for 4 weeks, and the mice were sacrificed 6 weeks after tumor cell injection. The IVIS imaging was conducted weekly after tumor implantation (Fig. 2B, left). Figure 2B (right) shows the bioluminescence imaging of tumor volume over time. Results showed a gradual increase in tumor volume in the control group (Fig. 2B, right) compared with the remaining groups. Overall, there was significant day and treatment-by-day effect (type III test, $P < 0.0001$). However, the differences in IVIS imaging were not significant on day 0 and day 7. On day 14, 21, and 28, the differences between the control and the capecitabine group and between the control and ursolic acid + capecitabine group were significant. Figure 2C showed the effect of capecitabine and ursolic acid on tumor volume measured on the last day of the experiment at autopsy. Capecitabine significantly reduced tumor volume ($P = 0.0335$). There was a trend that ursolic acid also reduced tumor volume, but the effect was not statistically significant ($P = 0.11$). Tumor volume of the mice treated with ursolic acid plus capecitabine was significantly smaller than that of the vehicle control group. The linear model showed that capecitabine significantly decreases tumor weight ($P = 0.021$). The effect of ursolic acid is not statistically significant ($P = 0.12$), possibly due to small sample size. Compared with the vehicle control, tumor weight of the combination treatment was significantly smaller ($P = 0.027$; Fig. 2D).

Ursolic acid inhibits distant organ metastasis and ascites

We also evaluated whether ursolic acid alone or in combination with capecitabine can inhibit ascites formation and distant organ metastasis in nude mice. We monitored the animals every day for ascites tumors by observing the increase in belly size. Vehicle-treated mice developed ascites tumors 27 days after tumor cell implantation, and the presence of ascites tumor was confirmed at the time of death. There were no statistically significant differences in ascite incidence rate among treatment groups (Fisher exact test $P = 0.57$) due to small sample size (Fig. 3A). On day 42, mice were sacrificed and examined for the presence of metastases. The examination showed that colon cancer metastases developed in the liver, intestines, lungs, and spleen of vehicle-treated mice. Ursolic acid alone inhibited metastasis to some extent, as did capecitabine in most organs; however, when they were used in combination, the inhibition of metastasis was maximal in these mice (Fig. 3B).

Ursolic acid is bioavailable in serum and CRC tumor tissue

Because ursolic acid inhibited tumorigenesis in nude mice, we therefore investigated whether ursolic acid is bioavailable in the serum and the CRC tumor of the mice. HPLC results showed that a significant amount of ursolic acid was available in the serum of the mice treated with ursolic acid alone (480 ng/mL) or with ursolic acid plus capecitabine (477 ng/mL). The presence of capecitabine did not affect the level of ursolic acid concentrations in serum. Ursolic acid was also found in CRC tumors (Fig. 4A). Although the amount of ursolic acid in CRC tumor was lesser than that in the serum of mice, it was comparable. Purified ursolic acid was used as a control.

Ursolic acid inhibits CD31 and Ki-67 expression

The Ki-67–positive index is used as a marker for cell proliferation, and the CD31 index is a marker for microvessel density. We examined whether ursolic acid and capecitabine can modulate these biomarkers. We found that both ursolic acid alone and capecitabine alone
downregulated the expression of Ki-67 in CRC tissue and the combination of the 2 was more effective (Fig. 4B). Similarly, we found that both agents alone reduced CD31 expression, compared with the control group, and the combined ursolic acid plus capecitabine was the most effective (Fig. 4B). The differences in expression of Ki-67 and CD31 between each of the 3 active treatments and the vehicle control were statistically significant (Bonferroni adjusted P value, <0.007).

**Ursolic acid inhibits NF-κB in CRC tumors**

NF-κB is known to be overexpressed in CRC tumors (34). Therefore, we determined whether ursolic acid could inhibit activated NF-κB in the CRC tumors. We found that ursolic acid inhibited activated NF-κB in these tissues and that combined ursolic acid plus capecitabine was more effective (Fig. 5A). The differences in expression of NF-κB between each of the 3 active treatments and the vehicle control were statistically significant [Bonferroni adjusted (for all 6 pairwise comparisons), P value <0.001).

**Ursolic acid inhibits β-catenin expression in CRC tumors**

Overexpression of nuclear β-catenin in CRC is known and found to be strongly associated with metastasis (35). Therefore, we investigated to determine whether ursolic acid inhibits the expression of β-catenin in tumors. We observed that the treatment of ursolic acid resulted in suppression of β-catenin. The combination of ursolic acid and capecitabine treatment results in greater inhibition of β-catenin than each agent alone [Bonferroni adjusted (for all 6 pairwise comparisons) P value <0.01; Fig. 5B].

Figure 4. A, ursolic acid is bioavailable in the serum of nude mice and in CRC tumors. Animals were fed with ursolic acid 4 hours before being sacrificed. Mice were anesthetized and blood was collected by cardiac puncture, and then the mice were sacrificed humanely. HPLC analysis was conducted to determine ursolic acid bioavailability in the serum and CRC tumors. B, immunohistochemical analysis of the proliferation marker Ki-67 and CD31 microvessel density in tumor tissue. Percentages indicate positive staining for the given biomarker.
Ursolic acid suppressed the activation of transcription factor STAT3
Next, we examined whether ursolic acid modulates the activation of STAT3 overexpression because STAT3 is found to be constitutively active (36) in CRC tumors. We found that ursolic acid and capecitabine alone moderately suppressed the activated STAT3 by inhibiting phosphorylation at Tyr705; however, ursolic acid in combination with capecitabine markedly inhibited its phosphorylation (Fig 5C, left). Thus, inhibition of this transcription factor by ursolic acid could be associated with enhancement of capecitabine-induced CRC tumor regression.

Ursolic acid suppressed the activation of EGFR
Most of the CRCs are characterized with overexpression of EGFR and predicted with high risk of metastasis and recurrence (37). Targeting EGFR seems to be a promising approach for the CRC treatment. In present experiment, moderate inhibition of EGFR activation was observed by either ursolic acid alone or capecitabine alone or by combination (Fig 5C, right).

Ursolic acid inhibits the expression of various biomarkers
We also examined the expression of various biomarkers in CRC tumor samples by Western blot (Fig. 5D). The results showed that ursolic acid alone or capecitabine suppressed the expression of cyclin D1, c-myc (involved in proliferation), Bcl-2, Bcl-xL, and survivin (involved in cell survival). Ursolic acid in combination with capecitabine inhibited proteins like cyclin D1, c-myc, and Bcl-2 more profoundly than either drug alone (Fig. 5D). However, inhibition of survivin and Bcl-xL was not prominent in combination compared with either ursolic acid alone or capecitabine alone.

Immunohistochemical analysis showed reductions in the expression of MMP-9 and VEGF (involved in invasion and metastasis) in tumors from the ursolic acid alone group (Bonferroni adjusted \( P \) value = 0.053) and those from the capcitabine alone group (Bonferroni adjusted \( P \) value <0.001) compared with the control group (Fig. 6A). Results also indicated that the combination of ursolic acid and capcitabine was more effective than treatment with either ursolic acid alone (Bonferroni adjusted (for all 6 pairwise comparisons) \( P \) value <0.001) or capcitabine alone (Bonferroni adjusted \( P \) value = 0.053). Western blotting results also confirmed that expression of proteins involved in invasion and metastasis like MMP-9, VEGF, and ICAM-1 decreased by either drug compared with the control treatment. However, in combination with capcitabine, decrease of MMP-9, VEGF, and ICAM-1 expression by ursolic acid was more prominent compared with either agent in CRC tumor tissues (Fig 6B).
Ursolic acid upregulates tumor suppressor proteins

The activation of oncogenes and inactivation of tumor suppressor genes have been implicated in the development of many human and animal malignancies. Therefore, we determined whether ursolic acid modulated p53. We found that p53 and its downstream target p21 was upregulated by either ursolic acid or capecitabine treatment. Combination of ursolic acid and capecitabine enhance the level of p53 and p21 (Fig 6C).

Discussion

Although capecitabine as a chemotherapeutic drug is frequently used in patients with stage III CRC after surgery to remove their tumors, this agent has limited efficacy and has side effects (38). Therefore, in this study, we investigated whether ursolic acid derived from various fruits and vegetables has potential either alone or in combination with capecitabine treatment. Combination of ursolic acid and capcitabine enhance the level of p53 and p21 (Fig 6C).

Ursolic acid upregulates tumor suppressor proteins

This is the first report to show that ursolic acid can suppress CRC growth and enhances the effect of capcitabine in an orthotopic mouse model. Whether ursolic acid affects the concentration of capecitabine or its analog is not known at present. However, ursolic acid is known to inhibit cytochrome P450 1A2 activities in human liver microsomes (39). Although one study reported that ursolic acid enhanced the postirradiation responses to gamma radiation and decreased undesirable radiation damage to hematopoietic tissue after radiotherapy (9), this study did not describe the mechanism of action. In our study, the inhibition of tumor growth by ursolic acid alone or in combination with capcitabine seemed to be because of the suppression of multiple biomarkers. Ursolic acid inhibited proliferative proteins, cyclin D1 and Ki-67, indicating one of the probable mechanisms of the antitumorigenic potential of ursolic acid. EGFR overexpression has also been shown in some of the CRC tumor cells (40), which was downregulated by ursolic acid in the present study. Previously Shan and colleagues (41) showed that ursolic acid suppressed the phosphorylation of EGFR, ERK1/2, p38 MAPK, and JNK in colorectal cancer cells, which was correlated with its growth inhibitory effect.

We also found that ursolic acid alone can inhibit CRC metastasis to different organs and also suppress CRC-induced ascites. The inhibition of metastasis could be due to the suppression of MMP-9 and VEGF. Ursolic acid indeed decreased angiogenesis, as indicated by inhibition of CD31, a marker for microvessel density, and VEGF. It has been reported earlier that ursolic acid inhibited tumor-associated capillary formation by reducing VEGF, and proinflammatory cytokines in C57BL/6 mice induced by highly metastatic B16F-10 melanoma cells (42). Ursolic acid can also
downregulate MMP-9, which is in agreement with previous reports (18). Inhibition of metastasis by ursoic acid may also be due to CXCR4, a chemokine receptor closely linked to metastasis and which has been reported to be downregulated by ursoic acid (43).

Our finding that ursoic acid downregulated expressions of MMP-9 and ICAM-1, which are involved in tumor invasion, were significantly suppressed in tumor tissues from ursoic acid-treated mice. It has been shown that MMP-9 and ICAM-1 are overexpressed in patients with CRC (44, 45), and it has also been observed that negative MMP-9 expression levels correlate with longer survival time in patients with CRC (44). Finally, our results showed that, in CRC, ursoic acid inhibited several antiapoptotic proteins regulated by NF-κB, including survivin, Bel-2, Bel-xL, and cIAP-1. These results suggest that ursoic acid enhances the apoptotic effect of capectabine by inhibiting antiapoptotic proteins in CRC cells.

Overall, our results suggest that ursoic acid has significant potential for the treatment of CRC, and it can further enhance the effects of capectabine by inhibiting NF-κB and associated biomarkers that are involved in proliferation, angiogenesis, invasion, and metastasis. On the basis of these results, further studies are required in patients to explore the potential of ursoic acid as an anticancer agent.

Disclosure of Potential Conflicts of Interest

Dr. B.B. Aggarwal is the Ransom Horne, Jr, Professor of Cancer Research. No potential conflicts of interest were disclosed by the other authors.

References


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Provided technical help in tumor implantation, bioluminescence imaging, tumor collection, and in general animal handling during the in vivo experiment: A.A. Deorukhkar
Organized the imaging schedule and helped in the data acquisition of in vivo bioluminescence imaging: P. Diagaradjane

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