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*Research Paper*

# Chemoprevention of Colon Carcinogenesis by Oleanolic Acid and Its Analog in Male F344 Rats and Modulation of COX-2 and Apoptosis in Human Colon HT-29 Cancer Cells

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***Purpose.*** To assess the chemopreventive effect of oleanolic acid (ONA) and its synthetic analog 18α-olean-12-ene-3β-23,28-triol (OT) on azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) in F344 rats and understand anti-inflammatory properties and apoptosis effects in HT29 colon cancer cells and Raw 264.7 macrophage cell lines.
***Methods.*** Five week-old male F344 rats were fed a control diet or experimental diets containing two doses of ONA (750 and 1,500 ppm) and OT (250 and 500 ppm). After 1 week, all animals were *s.c.* injected with AOM (15 mg/kg body weight, once weekly for 2 weeks). At 14 weeks of age, all rats were killed and colons were evaluated for ACF. Cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS) expressions and apoptosis were assessed in cell lines exposed to OT using western blots and 4',6-diamidino-2-phenylindole staining.
***Results.*** Administration of ONA and OT inhibited mean colonic ACF and multi-crypt AC/foci in a dose dependent manner ($p<0.001$–$0.0001$). OT blocked the COX-2 expression induced by phorbol 12-myristate 13-acetate in a dose-dependent manner and induced apoptosis in HT-29 cancer cells, and suppressed iNOS activation in RAW264.7 macrophages.
***Conclusions.*** ONA and OT possess chemopreventive activity against colon carcinogenesis in rat and OT inhibits the COX-2 and iNOS and induces apoptosis in cell lines.

**KEY WORDS:** chemoprevention; colon cancer; COX-2; iNOS; triterpenoids.

## INTRODUCTION

Colon cancer is the third most common cancer in the US, prevalent in both men and women. Globally, colon cancer is the fourth most common cancer in men and the third most common cancer in women. In the USA, as per the statistics of the National Cancer Institute of 2007, there are 112,340 new cases of colon cancer, 41,420 new cases of rectal cancer and 52,180 deaths from both cancers combined. Therefore, attention has focused on developing natural/synthetic agents to reduce the mortality from colorectal cancers. Triterpenoids, which owe their origins to plant molecules (particularly medicinally important plants) are used as medicines in Asian countries (1).

Interest in elucidating the biological roles of triterpenoid compounds has recently grown in terms of understanding the hepatoprotectory, analgesic, antitumor, anti-inflammatory and immunomodulatory effects of these compounds (2,3). Triterpenoid saponins are broken down in the gut to release triterpenes, which are absorbed and integrated in cell membranes leading to modulation of signaling mechanisms of various genes de novo. Oleanolic acid (ONA, 3α-3-hydroxyolean-12-en-28-oic acid), pentacyclic triterpene acid, is one such triterpene isolated from various medicinal plants. Oleanolic acid is relatively non-toxic and has been used in cosmetics and health products (3).

Synthesizing the analogs of triterpenoids improves the potency and proves to better anti-inflammatory and anti-carcinogenic agents than natural ones. Synthetic triterpenoids suppress the formation of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 (4), the known enhancers of carcinogenesis, in the cellular response to various inflammatory cytokines (5–10). Hence, ONA and its new synthetic analog 18α-olean-12-ene-3β-23,28-triol (OT) are tested for their chemopreventive and anti-inflammatory properties in colon cancer (Fig. 1a). These agents were tested in the animal model of colon cancer for chemopreventive efficacy using colonic precursor lesions, aberrant crypt foci, as a surrogate biomarker. To understand the anti-inflammatory effects of OT, we utilized HT29 human colon cancer cells and Raw 264.7 macrophage cells and studied the inhibitory effect on COX-2 and iNOS and induction of apoptosis.

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## MATERIALS AND METHODS

### *In Vivo* Experiments

#### *Animals, Diet and Care*

All animal experiments were performed in accordance with the institutional guidelines of the American Council on Animal Care. Male F344 rats were obtained from Charles River Laboratories (Kingston, NY, USA) and housed under standardized conditions (21°C, 60% relative humidity, 12 h light/12 h dark cycle, 20 air changes per hour). Animals were fed a standard laboratory rodent chow and drinking water until initiation of the experiment. Experimental diets were prepared based on modified AIN-76A containing 5% corn oil by weight (American Institute of Nutrition). ONA was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and OT was kindly provided by the National Cancer Institute repository. The experimental diets contained 0.075% (750 ppm) or 0.15% (1,500 ppm) of ONA and OT 0.025% (250 ppm) or 0.05% (500 ppm) for animal bioassay. Diets were prepared once a week and stored at 4°C until used. Rats were allowed *ad libitum* access to the respective diets and water.

#### *Determination of the Maximum Tolerable Dose (MTD) of ONA and OT*

The purpose of this MTD study was to determine the tolerable dose of ONA and OT in F344 rats. MTD is defined as the highest dose that causes no more than 10% weight decrement, compared to the appropriate control diet group, and does not produce mortality or any clinical signs of toxicity that would be predicted to shorten the natural life span of the animal. At 6 weeks of age, groups of male F344 rats (six rats/group) were fed the AIN-76A diet containing 0%, 0.025%, 0.05%, 0.075%, 0.1% and 0.15% of ONA and 0.005%, 0.01%, 0.02%, 0.035% and 0.05% of OT. Body weights were recorded once weekly for 6 weeks. All animals were killed after 6 weeks and the organs were examined grossly for any abnormalities.

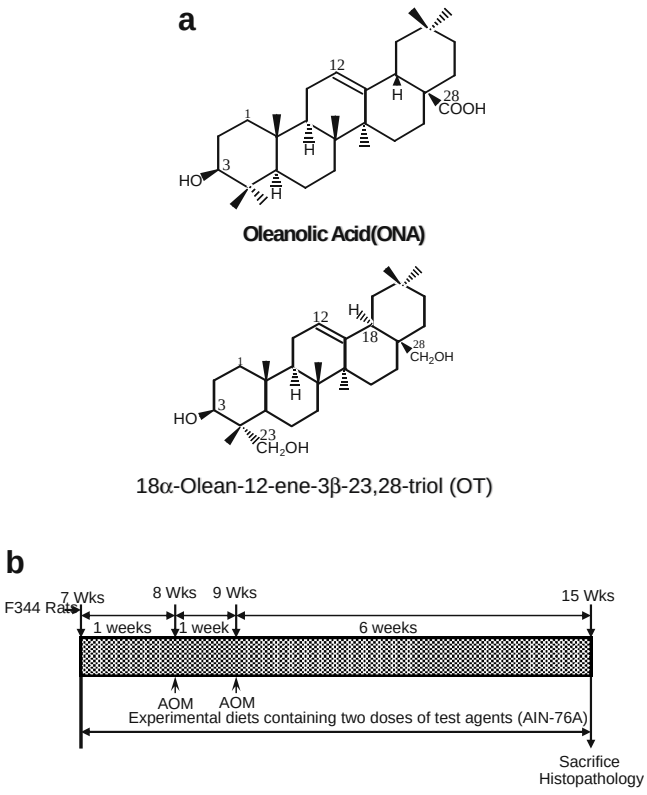


**Fig. 1.** **a** Chemical structures of ONA and OT where OT is a synthetic analog of ONA. Incorporation of hydroxyl molecules at 23rd and 28th position in OT has an effect on its solubility/absorption. **b** Experimental design for evaluation of the preventive potential of ONA and its analog on AOM-induced colonic ACF formation in F344 male rats.

#### *Experimental Design for Efficacy of ONA and OT*

The experiment was designed to evaluate the efficacy of ONA and OT against the chemically-induced colon carcinogenesis. The dose selection ONA and OT was based on our MTD study. At 7 weeks of age, groups of rats ($n$=18 rats per group; azoxymethane (AOM) treated 12 rats plus vehicle treated six rats) were fed either the control diet or experimental diet containing of ONA 0.075% (750 ppm) or 0.15% (1,500 ppm) and OT 0.025% (250 ppm) or 0.05% (500 ppm). At 8 weeks of age, rats intended for carcinogen treatment were injected *s.c.* with AOM (Midwest Research Institute, KA, USA) at a dose rate of 15 mg/kg body weight once weekly for 2 weeks, and those intended for vehicle treatment received an equal volume of normal saline. These dietary regimens were continued until termination of the experiment, i.e., 6 weeks after the second AOM treatment (Fig. 1b). Rats were killed by $CO_2$ euthanasia and all organs were examined grossly. For evaluation of colonic pre-neoplastic lesions, they were cut open longitudinally and cleared the colonic contents and then fixed flat with mucosa side up between filter papers in 10% buffered formalin. Colons were evaluated for aberrant crypt foci (ACF).

#### *Quantification of ACF*

Topographical analysis of the colonic ACF was performed after a minimum of 24 h of fixation, according to Bird (11). Colons were cut in to 2-cm sections and stained with 0.2% methylene blue solution for ~5 min, placed mucosal side up on a microscopic slide and viewed under a light microscope. The total number of ACF and number of crypts in each foci in the entire colon was determined from the distal (taken as 0 cm) to the proximal end. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, increased distance from lamina to basal surfaces of cells and easily discernible pericryptal zone. The parameters used to assess the aberrant crypts were occurrence and multiplicity. Aberrant crypt multiplicity was determined as the number of crypts in each focus and categorized as containing one, two, three, and four or more aberrant crypts/focus.

### *In Vitro* Experiments

#### *Cell Culture*

Human colon cancer HT29 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and

maintained in McCoy's 5A medium containing L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum, 100 units per milliliter penicillin and 100 μg/ml streptomycin. The mouse monocytic-macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, Gaithersburg, MD, USA) containing 10% heat-inactivated fetal bovine serum and antibiotics (100 units per milliliter of penicillin-G and 100 μg/ml streptomycin). Cells were incubated at 37°C in a humidified atmosphere with 5% $CO_2$ and sub-cultured after trypsinization (0.5% trypsin/2.6 mM ethylenediaminetetraacetic acid (EDTA)). For all experiments, cells were seeded at $1\times10^6$ cells in culture dishes (100 mm) and grown to 60–70% confluence. To study the apoptosis, COX-2 and iNOS expressions, we used various sub-toxic doses of OT ranging from 0 to 60 μM.

*3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay*

Cytotoxicity was measured using a standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay by plating calculated number of HT29 cells in 96-well plates and treated with various concentrations of OT for 24 h. The color formed was then quantified by spectrophotometric method using an enzyme-linked immunosorbent assay plate reader at a wavelength of 570 nm. The amount of color produced was directly proportional to the number of viable cells and a quantification of changes in proliferation. Data were derived from at least three independent experiments (in triplicate) for the OT. The percentage of cell viability was calculated using the equation: [mean optical density (OD) of treated cells/mean OD of control cells]×100.

*DAPI Staining*

Cells were harvested after treatment with ONA synthetic analog, OT (0–60 μM) and washed once with phosphate-buffered saline (PBS), then resuspended in PBS containing 0.1% Triton X and incubated for 10 min on ice. Cells were spun down and resuspended at 5,000 cells per microliter in 4% PBS buffered paraformaldehyde solution containing 10 μg/ml 4'6-diamidino-2-phenylindole (DAPI, Sigma). 10 μl of this suspension were placed on a glass slide and covered with a cover slip. The morphology of the cells nuclei was observed using a fluorescence microscope (Olympus) at excitation wavelength 350 nm. Nuclei were considered to have the normal phenotype when glowing bright and homogenously. Apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. More than 150 cells were counted and the percentage of apoptotic nuclei determined.

*Western Immunoblotting*

Briefly, HT29 cell monolayers were stimulated with phorbol 12-myristate 13-acetate (PMA) for COX-2, and RAW 264.7 cell monolayers were stimulated with lipopolysaccharide (1 ng/ml) for iNOS and co-incubated with different doses of OT (0–40 μM) for 24 h. Cells exposed to OT (0-40 μM) were harvested by gentle scraping and/or centrifugation and lysed in ice cold lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, and protease inhibitor cocktail], tested for COX-2 and iNOS protein expressions using antibodies, procured from Santa Cruz biotechnology (Santa Cruz, CA, USA). After a brief vortexing, the lysates were clarified by centrifugation at 12,000×*g* for 15 min at 4°C, and protein content was measured by the Bio-Rad Protein Assay reagent (Hercules, CA, USA). An aliquot (50 μg protein/lane) of the total protein was separated by 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking in 5% milk, primary antibody [1:500, in Tris-buffered saline–Tween 20 solution] was incubated overnight at 4°C followed by washing, secondary antibody (horse radish peroxidase conjugated) incubation for 1 h, and visualized with SuperSignal® West Pico Chemiluminescence Substrate (Pierce, Rockford, IL, USA). The bands were captured on Ewen Parker, Blue sensitive X-ray films.

**Statistical Analysis**

Data is reported as mean±SE. Statistical differences between control and treated groups were evaluated using unpaired *t*-test with Welch's correction. Differences between groups are considered significant at $p<0.05$.

## RESULTS

**MTD and General Observations of *In Vivo* Experiments**

Rats were exposed to five different dietary doses ranging from 250 to 1,500 ppm of ONA and 50 to 500 ppm of OT for 6 weeks to determine any effect on body weight gain, eating habits and/or overt toxicity. Our results suggest that ONA and OT at maximum doses (1,500 and 500 ppm respectively in diet) do not produce any retardation in body weight gain and observable toxicities. Based on these dose tolerability results, we have tested the 750 and 1,500 ppm ONA and 250 and 500 ppm of OT in diet to assess the chemopreventive effects of these agents against the colonic ACF formation. In the ACF study, the initial body weight before interventions with control or ONA and OT diets was 98±1.2 g (mean±SE). At the time of termination, there was no significant difference in body weight of control and treated rats (data not shown). The food intake of animals in the experimental groups did not show any variation.

**Inhibitory Effect of ONA and OT Against the AOM-Induced Colonic ACF**

In rats fed the control diet, AOM-induced 89.5±7.9 (mean±SEM) colonic ACF containing 22% of one crypt foci; 42% of two crypt foci; 18% of three crypt foci and 18% of four or more crypt foci (Fig. 2a). Rats fed ONA and OT diet showed significantly lower number of total mean ACF/colon (36–52%, $p<0.01$ to $p<0.001$ and 32–48% $p<0.01$ to $p<0.001$ respectively) when compared to rats which were fed control

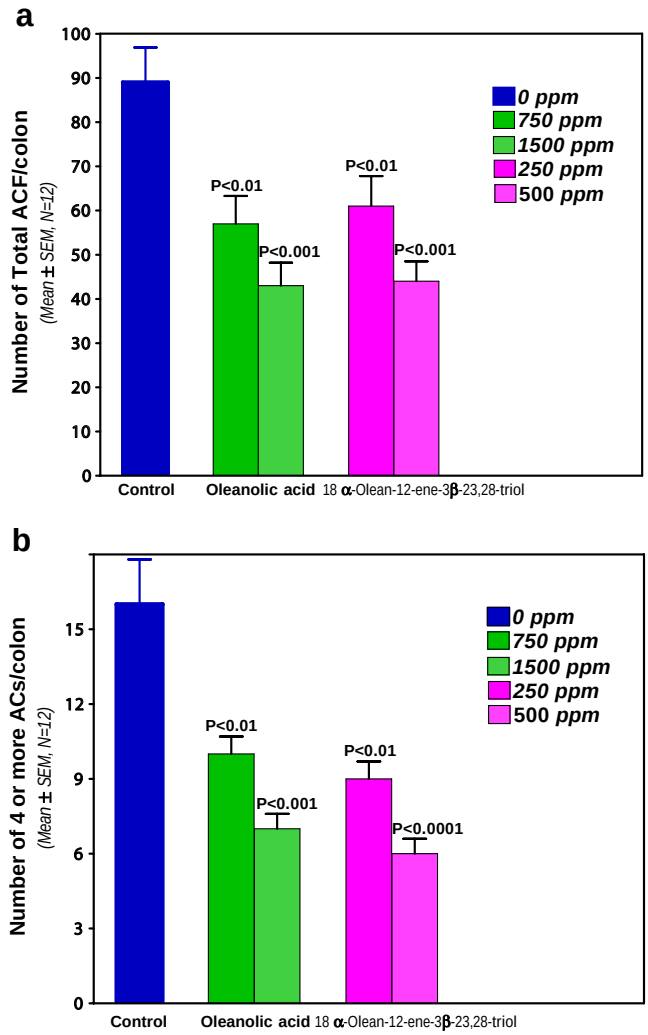


**Fig. 2.** **a** Effect of ONA and its analog OT on AOM-induced total colonic ACF formation in male F344 rats. A significant decrease in number of total colonic ACF/colon is observed in rats fed with ONA and OT diets. **b** Effect of ONA and OT on AOM-induced colonic ACF containing four or more aberrant crypts in male F344 rats. ONA and OT diet fed rats showed a significant decrease in four or more aberrant crypts.

diet (Fig. 2a). Importantly, aberrant crypts containing multi-crypt foci (four or more) were reduced significantly (47–67%, $p<0.001$ and 40–60%, $p<0.01$; Fig. 2b) in rats fed the ONA and OT diet, respectively. Although both ONA and OT diets induced significant inhibition of AOM-induced colonic ACF, our results show ONA synthetic analog OT to be effective with lower dose concentrations compared to ONA and both the drugs showed a dose-response effect.

### Effect of OT on Cell Growth of HT29 Human Colon Cancer Cell Line

HT29 cells were exposed for 24 h to OT at concentrations varying from 0 to 80 μM. As shown in Fig. 3a, cell growth inhibition was observed in a dose-dependent manner. At ~40 μM, OT induced 50% inhibition of cell growth after 24 h of exposure. At 80 μM or above concentrations, OT induced significant toxicity in HT29 cells. All the *in vitro* experiments with OT were performed using up to 40 μM concentration except for DAPI staining.

### OT Induces Apoptosis in HT29 Cells

We observed the apoptotic bodies by staining with DAPI. The population of apoptotic cells was significantly ($p<0.0001$) enhanced in OT treated HT29 cells. As shown in Fig. 3b, OT induced apoptosis in HT29 cells in a dose-dependent manner (12.7% in 20 μM, 27% in 30 μM, 48% in 50 μM and 55% in 60 μM) *versus* untreated cells.

### Down Regulation of COX-2 and iNOS in HT29 Cells and Raw 264.7 Cells Respectively Upon OT Treatment

Strong dose-dependent decrease in PMA-induced COX-2 protein expression levels (up to 50–60%) was seen in OT (20–40 μM) treated HT29 cells (Fig. 4). Equally strong

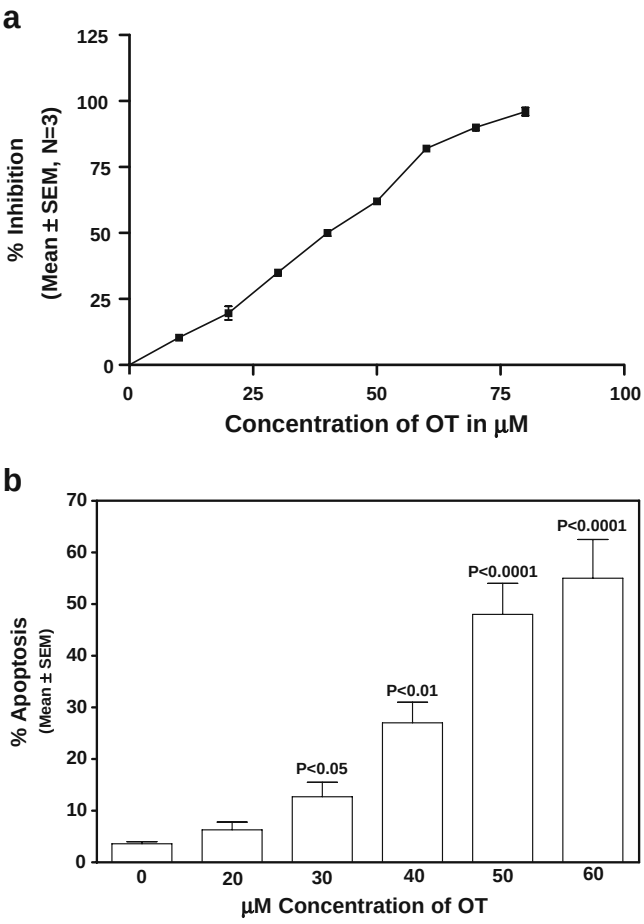


**Fig. 3.** **a** Effect of OT after 24 h of treatment on HT29 cell proliferation as measured by MTT assay. The color formed due to enzyme activity on addition of enzyme substrate is read at 570 nm in micro plate reader. $IC_{50}$ is calculated based on 50% inhibition of cell proliferation at a specific concentration of OT. Each *dot* in the graph represents the mean of three independent experiments. **b** Detection of apoptosis in OT treated HT29 cells by DAPI staining. Live cells stain uniformly blue and can be distinguished from apoptotic cells which exhibit dark blue dots of condensed chromatin.

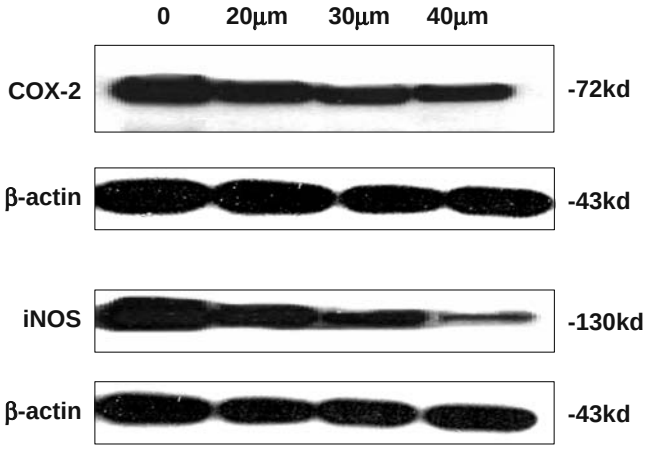


**Fig. 4.** Western blot analysis of HT29 and Raw 264.7 cells treated with OT. Cells grown to 70–80% confluence were exposed to OT for 24 h and lysates were collected to identify the effect of this agent on inflammatory markers COX-2 and iNOS.

decrease was observed in iNOS protein expression levels with high dose OT treatment in Raw 264.7 macrophage cell lines (up to 80%; Fig. 4). Relative to control, OT treatment substantially reduced inflammatory markers, COX-2 and iNOS (Fig. 4).

## DISCUSSION

Diet has been recognized as an important modifiable risk factor for colon cancer for decades. Specifically, a consistent inverse association between diets rich in plant foods and colon cancer incidence has been identified through epidemiologic studies (12–14). Phytochemicals, such as triterpenoids present in plant foods have been suggested to have health benefits. It is known that the triterpenoid compounds have anti-inflammatory properties and that ONA, in particular, could inhibit certain types of cancers in animal models (15–18). Synthetic triterpenoids are more potent than natural plant-derived triterpenoids for the prevention and treatment of cancers based on bioavailability by oral administration. In this process, OT, a synthetic analog of ONA, is synthesized and studied for its anti-carcinogenic properties and anti-inflammatory actions using *in vivo* and *in vitro* models of colon cancer. Further, OT's chemopreventive potential was observed in comparison to ONA in well-established rat colon carcinogenesis using ACF as end point surrogate biomarker for chemopreventive efficacy.

In the present study administration of ONA and OT in the diet significantly reduced AOM-induced total colonic ACF formation and multicrypt aberrant crypt growth. Previous studies have established that ACF containing four or more aberrant crypts correlate with colon tumor outcome. In this context, our earlier studies with naturally occurring agents, such as curcumin (19), caffeic acid ester (20) β-ionone (21), and diosgenin (22) significantly suppressed AOM induced colon ACF and colon adenocarcinoma in male F344 rats. It is important to note that ONA and OT suppress the carcinogen induced colonic preneoplastic lesions significantly with the dietary doses without any toxicity. This confirms the safe usage of ONA and OT at the doses we evaluated in the bioassay. In the present study, administration of ONA provided up to 52% inhibition of AOM-induced total ACF formation, and suppression of four or more crypts growth up to ≥66%, which clearly suggests the potential colon tumor inhibitory properties of ONA (Fig. 2a, b). ONA was previously tested by other investigators (23) and found to be effective in reducing the ACF in intestine of F344 rats with a dose of 200 ppm, whereas the experimental design is different from the one employed in this present study and the ONA used by these investigators was isolated from plants and not a pure chemical. OT could inhibit up to 48% of total AOM-induced ACF formation and importantly it could also suppress the formation of four or more crypts up to 60% with very low doses, compared to those of ONA (Fig. 2a, b). ONA has been reported to show anti-inflammatory and anti-tumor promoting effects in an *in vivo* short-term assay (24). Our results on ONA, is in agreement with previous reports suggesting potential colon carcinogenesis inhibitory properties in rats (25). A study which evaluated the chemopreventive activities of various triterpenoids *in vivo* on various cancer models has explained the poor absorption in the gut (26–29) and this can be the reason why ONA needs to be given in high doses compared to its analog OT. ACF inhibition by OT with very low doses, compared to ONA, explains its better absorption than ONA. OT at doses 500 ppm lesser than that of 1,500 ppm ONA showed similar efficacy for ACF inhibition and proves its potential as a better chemopreventive agent (Fig. 2a, b).

It has been reported that triterpenoids can induce apoptosis in hepatocellular carcinoma cell lines, liver cells, a cholangiocarcinoma cell line, a pancreatic cancer cell line, and a melanoma cell line (30,31). In the present study, OT treatment has induced significant apoptosis of HT29 colon cancer cells (Fig. 3b). This observation confirms the apoptotic property of OT similar to the naturally-occurring triterpenoids. Previous reports of others and ours show significant evidence indicating that the processes of inflammation and carcinogenesis share common mechanisms (3,5–7,32–35). Past studies have reported the anti-inflammatory, anti-tumor promoting properties of the several natural triterpenes like ONA, ursolic acid, lupeol and glycyrrhetinic acid (36,37). Hence, we evaluated the ability of ONA analog OT to block de novo synthesis of iNOS and COX-2 in Raw 264.7 and HT29 cell lines. In the present study, OT has been found to possess anti-inflammatory properties, mediated via inhibition of COX-2 and iNOS (Fig. 4). These findings suggest that the anti-inflammatory activities of OT are probably mediated by reducing the effect of mediators, such as COX-2 and iNOS, and weakening the inflammatory action of these mediators. It has been well established that COX-2 inhibitors such as celecoxib provides significant protection against the colon cancer in both preclinical and clinical levels (38,39). However, long-term use of COX-2 inhibitors have unwanted side effects and thus development of plant based agents such as ONA and OT, which block the expression of COX-2 and iNOS without having any toxicity, may provide the advantage. Furthermore, the potential of OT to act as a chemopreventive agent for colorectal carcinomas needs to be evaluated in animal models in long term tumor studies. Additional studies on the pharmacokinetics and toxicology of OT are now critically needed before it can be considered for any clinical trials. Future studies *in vitro* will determine the

exact molecular mechanism involving COX-2 and iNOS and pharmacokinetic profiles of these compounds.

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