Chemoprevention of Colon and Small Intestinal Tumorigenesis in APC\textsuperscript{Min/+} Mice by Licofelone, a Novel Dual 5-LOX/COX Inhibitor: Potential Implications for Human Colon Cancer Prevention

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Abstract

Preclinical and clinical studies suggest that 5-lipoxygenase (5-LOX), such as COX-2, is a potential target for colon cancer inhibition and, in part, contributes to cardiovascular side effects associated with COX-2 inhibitors. Experiments were designed to assess the chemopreventive effects of a novel dual 5-LOX/COX inhibitor, licofelone \{6-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl\} acetic acid, in APC\textsuperscript{Min/+} mouse intestinal tumorigenesis. Six-week-old male and female APC\textsuperscript{Min/+} mice (n = 10 per group) were fed with control American Institute of Nutrition-76A diet or diets containing 150 or 300 ppm licofelone for 14 weeks (~100 days), and intestinal tumors were evaluated for tumor multiplicity and size. Licofelone significantly inhibited total intestinal tumor multiplicity and size in a dose-dependent manner (P < 0.0001; mean tumors for 0, 150, and 300 ppm: 48.8, 17, and 8, respectively, in male mice; and 34.3, 8.8, and 5.5, respectively, in female mice). Licofelone at high dose showed more than 83% (P < 0.0001) tumor inhibition in both genders of mice. One hundred and fifty and 300 ppm licofelone resulted in 86% to 97% inhibition of polyps having size greater than 2 mm. One hundred and fifty and 300 ppm licofelone caused more than 72% and 100% inhibition of colonic tumors, respectively. Importantly, in mice fed with licofelone, tumors showed significantly reduced proliferating cell nuclear antigen expression (70%, P < 0.0001), increased terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–positive cells (75%, P < 0.0001), and there was dose-dependent suppression of serum triglycerides (71%–83%, P < 0.0001), decreased inflammatory cytokines; and decreased COX and 5-LOX activities (57%–64%, P < 0.0001). Also, compared with 300 ppm celecoxib, 300 ppm licofelone provided better efficacy in suppressing tumor growth. These observations show that a novel dual 5-LOX/COX inhibitor dramatically suppresses small intestinal and colonic tumor formation in APC\textsuperscript{Min/+} mice. Cancer Prev Res; 4(12); 2015–26.

Introduction

Colon cancer is the third leading cause of cancer mortality for men and women in the United States (1). Globally, 1.1 million cases and 600,000 deaths are reported every year from colorectal cancers (CRC). Drug development has led to discovery of potential chemopreventive agents that are effective at the preclinical and clinical levels (2–7). Agents that target COX-2, such as celecoxib, are noteworthy because of their clinical efficacy in the prevention of polyp formation and for contributing to cardiovascular risk (7). Recent 5-year efficacy and safety analysis of adenoma prevention with celecoxib suggests a significant interaction between celecoxib treatment and cardiovascular and thrombotic events for those reporting a baseline history of atherosclerotic heart disease (7). Overall, targeting COX-2 for...
colorectal cancer prevention is still valid, but use of higher doses of COX-2 inhibitors in individuals at high risk for colon cancer and, more so, in those at high risk for atherosclerotic events suggests a need for new approaches for colon cancer prevention and treatment.

Several lines of evidence show that 5-lipoxygenase (5-LOX) and its molecular partner, 5-LOX activating protein (FLAP), are significantly overexpressed in colonic tumors harvested from preclinical and clinical samples (8–13). It is evident from recent studies that the 5-LOX metabolite leukotriene (LT) B4 (LTB4) and the cysteinyl leukotrienes (cys-LT)—LTC4, LTD4, and LTE4—may contribute to the development and progression of colon cancer (14, 15). Similar to COX-2, the expression and activity of 5-LOX have been found to be upregulated during colon carcinogenesis (16) and closely related to tumor size, depth, and vessel invasion (14). Thus, 5-LOX has been implicated in both inflammation and carcinogenesis (9–13). Hence, 5-LOX has been considered a potential target for colon cancer inhibition (17, 18).

Some effects of COX-2 inhibition include shunting of arachidonic acid (AA) into the 5-LOX pathway. There is evidence linking leukotrienes to cardiovascular disease (19), with increased urinary levels of LTE4 in patients with myocardial infarction and coronary artery disease (19). In this context, it is noteworthy that studies by Duffield-Lillico and colleagues show higher urinary levels of prostaglandin (PG)E metabolites and LTE4 in smokers. Smokers on celecoxib, however, showed shunting of AA into the 5-LOX pathway with increased levels of LTE4 (19). In addition, the 5-LOX pathway is abundantly expressed in arterial walls of patients with various stages of atherosclerosis (20), and 5-LOX also has been linked to atherosclerosis in some mouse models (21). Furthermore, 2 human genetic studies have correlated polymorphisms of the 5-LOX pathway with relative risk for myocardial infarction, stroke, and atherosclerosis (22, 23). In fact, therapies are being developed to target the 5-LOX pathway to reduce the risk of myocardial infarction (24, 25). The striking interrelationship of their biological functions suggest that molecules that are able to block both COX-2 and 5-LOX pathways may provide a promising approach to colon cancer prevention.

To date, several dual COX/5-LOX inhibitors have been designed and several compounds currently are undergoing clinical development as anti-inflammatory drugs (26). However, no data is available about their potential use as anticancer agents in colon cancer. It is important to evaluate the possible antitumorigenic effects of the dual COX/5-LOX inhibitor licofelone [16-(4-chlorophenyl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrroloizin-5-yl acetic acid] in APCMin/+ mouse intestinal tumorigenesis. Among dual COX/5-LOX inhibitors, licofelone is the molecule in the most advanced phase of clinical trials as an anti-inflammatory drug (27), and its safety and efficacy, in comparison with the nonsteroidal anti-inflammatory drugs (NSAID) naproxen and rofecoxib, have been reviewed (28, 29). In the following experiments, we show that licofelone is able to inhibit dramatically both small intestinal and colon tumorigenesis in APCMin/+ mice. Licofelone, as well as similar dual COX/5-LOX inhibitors, may represent a novel class of chemopreventive agents for colon cancer prevention and treatment.

Materials and Methods

Chemicals

Licofelone (Merckle Ratiopharm, GmbH; Fig. 1A) was kindly provided by the National Cancer Institute chemopreventive drug repository (Rockville, MD). Primary antibodies (monoclonal/polyclonal) to COX-2, 5-LOX, and proliferating cell nuclear antigen (PCNA), were from Santa Cruz Biotechnology; horseradish peroxidase–conjugated secondary antibodies were from Santa Cruz Biotechnology. Multi-Analyte ELISAarray kit was procured from SA Biosciences.

Breeding and genotyping of APCMin/+ mice

All animal experiments were conducted in accordance with the institutional guidelines of the American Council on Animal Care and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Oklahoma Health Sciences Center (OUHSC). Male APCMin/+ (C57BL/6) and female wild-type littermate mice were purchased initially from The Jackson Laboratory (Bar Harbor, ME) as founders, and our own breeding colony was established in the OUHSC rodent barrier facility and genotyped by the PCR method using primers (IMR0033 5'-GCC ATC CCT TCA CGT TAG-3', IMR0034 5'-TTC CAC TTT GGC ATA AGG C-3', IMR0758 5'-TTC TGA GAA AGA CAG TTA-3') according to vendor’s instructions. All mice were housed, 3 per cage, in ventilated cages under standardized conditions (21°C, 60% relative humidity, 12-hour light/12-hour dark cycle, 20 air changes per hour). All mice were allowed ad libitum access to the respective diets and automated tap water purified by reverse osmosis.

Experimental diets

All ingredients for the semipurified diets were purchased from Bioserv and stored at 4°C before diet preparation. Diets were based on the modified American Institute of Nutrition (AIN)-76A diet with slight modification. Licofelone was premixed with a small quantity of an experimental diet and then blended into bulk diet using a Hobart mixer. Both control and experimental diets were prepared weekly and stored in a cold room. Agent content in the experimental diets was determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to verify uniform distribution. In this study, we used 0, 150, or 300 ppm licofelone in the AIN-76A diet.

Efficacy studies in APCMin/+ mice

Both male and female APCMin/+ mice were used in the efficacy study. The experimental protocol is summarized in Fig. 1B. Five-week-old mice were randomized so that average body weights in each group were equal.
(10 APCMin/+ mice in each group) and mice were fed AIN-76A diet for 1 week. At 6 weeks of age, mice were fed control or experimental diets containing 0, 150, or 300 ppm licofelone until termination of the study. Body weight, food, and water consumption were monitored regularly and mice were evaluated weekly for signs of weight loss or lethargy that might indicate intestinal obstruction or anemia. After 100 days (~14 weeks) of exposure to licofelone diet, all mice were euthanized by CO2 asphyxiation, blood was collected immediately by heart puncture, and serum was separated by centrifugation and stored at -80°C until further analysis. This point in time was chosen to minimize the risk of mortality caused by severe progressive anemia, rectal prolapse, or intestinal obstruction, which usually occurs among Min mice at more than 20 weeks of age. After necropsy, the entire intestinal tract was harvested, flushed with 0.9% NaCl, and opened longitudinally from the esophagus to the distal rectum. The tissue was flattened on filter paper to expose the tumors and briefly frozen on dry ice to aid visual scoring of tumors. The number, location, and size of visible tumors in the entire intestine were determined under a dissection microscope (×5). All tumors were scored and subdivided by location (duodenal, jejunal, ileum, and colorectal) and size (>2 mm, 1–2, or <1 mm diameter). This procedure was completed by 2 individuals who were blinded to the experimental group and the genetic status of the mice. Colonic and other small intestinal tumors that required further histopathologic evaluation to identify adenoma, adenocarcinoma, and enlarged lymph nodes were fixed in 10% neutral buffered formalin, embedded in paraffin blocks, and processed by routine hematoxylin and eosin staining. In addition, multiple samples of tumors from the small intestines were harvested and stored in liquid nitrogen for analysis of COX-2 and 5-LOX activities and expression levels.

Figure 1. A, chemical structure of licofelone. B, experimental design for the evaluation of the chemopreventive efficacy of licofelone in APCMin/+ mice. Groups (10 mice per group) of mice were fed diets containing 0, 150, or 300 ppm licofelone from 6 weeks of age to the end of the experiment. The study was terminated after 100 days of exposure to the experimental diets (see Materials and Methods for more details). C and D, changes in body weights over time for male (C) or female (D) mice fed control diets and/or experimental diets containing 150 or 300 ppm licofelone. Statistically significant differences in body weight gain were observed between licofelone-treated and control groups. Data are presented as mean ± SEM. Statistical analysis was carried with ANOVA. Licofelone-treated animals were found to gain weight by the end of the study. E, MRI of the small intestine in APCMin/+ mice. Small intestines of the treated and control animals were scanned for polyps by MRI. Comparing images from pre- and post-Magnivest injection, polyps were identified as bright from accumulating Magnivest. Left and right panels show polyp images in control and treated mice, respectively.
Small intestinal and colon tumor count by MRI

At the end of the study, before euthanasia, MRI imaging was done using Tesla to assess the polyp count in live mice. Briefly, mice were anesthetized using 1.5% isoflurane at 0.7 L/min oxygen. A tail vein catheter was installed and the mouse was placed in a MR probe in a supine position. A 2-dimensional (2D) dataset was acquired (1-mm thick slices covering the rectum, colon, and small intestine, giving an in-plane resolution of 120 × 120 μm² for an acquisition time of 14 minutes) as well as a 3D dataset (volume acquired 30 × 18 × 36 mm³, pixel resolution of 120 × 200 × 555 μm³, for an acquisition time of 24 minutes). After the scans were acquired, Magnivist (gadopentate dimeglumine) was injected into the mice at a dose of 0.17 mmol/kg. Magnivist contrast agent is paramagnetic and decreases T1 and T2 relaxation times. In T1 weighted datasets, tissues that accumulate Magnivest appear bright in the case of polyps.

Serum triglycerides, packed cell volume

Triglycerides (TG) were determined in the nonhemolyzed serum with InfinityTM TGS liquid stable reagent (Thermo Scientific) as per the manufacturer’s instructions. For packed cell volume (PCV)/hematocrit measurement, blood was sampled by cardiac puncture with a 21-gauge needle attached to a 1-mL syringe and dispensed into a plastic microfuge tube while on ice. Microhematocrit tubes containing ammonium heparin were then placed in the microfuge tube and centrifuged in a hematocrit centrifuge for 5 minutes.

Apoptosis

Intestinal tumor tissues from licofelone-treated and -untreated mice were fixed in 10% formalin for 24 hours and then embedded in paraffin. Sections of 5-μm thickness were cut and mounted on slides, rehydrated, and stained using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method as previously described. TUNEL-positive cells were visualized by chromogenic staining with DAB and slides were counterstained with methyl green. Stained apoptotic epithelial cells (a minimum of 10 microscopic fields per section) were counted manually in a single-blind fashion.

Analysis of COX-1 and -2 and 5-LOX activity using radio-HPLC

Frozen intestinal polyps of male mice fed with 150 ppm of licofelone and/or control diet were homogenized using an ice-cold homogenizing buffer. Briefly, 150 μL of the reaction mixture containing 12 μmol/L \([^{14}C]\) AA (420,000 dpm), 1 mmol/L epinephrine, 1 mmol/L glutathione in 50 mmol/L phosphate buffer (pH 7.4), and 30 mg of protein from intestinal polyps were used for each assay. Similarly, 5-LOX activity was carried out in cytosolic fractions of tumor samples. After incubation at 37°C for 20 minutes, the reaction was terminated by adding 40 μL of 0.2 mol/L HCl. The COX-generated metabolites of AA were extracted with ethyl acetate (3 × 0.5 mL). The combined extracts were evaporated to dryness under N₂, dissolved in 1 mL of acetonitrile, and 10 μL were injected into a reverse-phase high-performance liquid chromatography (HPLC) system (Shimadzu Scientific Instruments) equipped with a Phenomenex C18 column (300 × 3.90 mm; pore size 10 μm). The [\(^{14}\)C]-PGs, [\(^{14}\)C]-TxB₂, [\(^{14}\)C]-PGE₂, and 5-[\(^{14}\)C]-HETE were eluted with a gradient solvent system containing solvent A: acetonitrile:water:acetic acid (35:65:0.1%) and solvent B: acetonitrile:water:acetic acid (65:30:0.1%). The eluted metabolites were monitored and quantitated by an IN/US Systems β-RAM radio HPLC detector.

Cell proliferation, COX-2, and 5-LOX expression

To evaluate the effect of licofelone, we assessed proliferating cell nuclear antigen (PCNA) expression in intestinal tumor tissue sections along with COX-2 and 5-LOX expressions levels by immunohistochemistry (IHC). Briefly, for PCNA, COX-2 and 5-LOX IHC staining, paraffin sections were deparaffinized in xylene and rehydrated through graded ethanol solutions to PBS. Antigen retrieval was carried out by heating sections in 0.01 mol/L citrate buffer (pH 6.0) for 30 minutes in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ in PBS for 5 minutes. Nonspecific binding sites were blocked using protein block for 20 minutes. Sections were then incubated overnight at 4°C with 1:300 dilutions of monoclonal/polyclonal primary antibodies against PCNA, COX-2, and 5-LOX (Santa Cruz Biotechnology). After several washes with PBS, the slides were incubated with secondary antibodies for PCNA, COX-2 and 5-LOX for 2 hours. The color reaction was developed with DAB. Nonimmune rabbit immunoglobulins were substituted for primary antibodies as negative controls. In addition, we have used blocking peptide to prove specificity for immunostaining in normal crypts and SI tumors for COX-2 expression, and we used a negative control without primary antibody for further confirmation (Supplementary Fig. S2). PCNA-positive cell scoring in the polyps was done by 2 investigators blinded to the identity of the samples (light microscopy at 400× magnification). Cells with a brown nucleus were considered positive. The proliferation index was determined by dividing the number of positive cells per polyp (upper, middle, and lower) and multiplying by 100.

COX-2, PGES-2, 5-LOX, and FLAP mRNA expression analysis by real-time and/or RT-PCR

Total RNA from tumor samples was extracted using RNA Kit for isolation of total cellular RNA (Ambion) as per the manufacturer’s instructions. Equal quantities of DNA-free RNA were used for reverse transcription reactions for making cDNA using SuperScript reverse transcriptase (Invitrogen). The real-time PCR was carried out in a 25-μL reaction volume using 3 μL of a 1:10 cDNA dilution containing SYBR Green master mix (BioRad) and primers for COX-2 (forward primer: 5’-GGATCTGCGCCAGCATCT-3’ and reverse primer: 5’-AGACCAGGCCACCCAGCAGAGA-3’), PGES-2 (forward primer: 5’-AAGACATGCTCCTCTGC-3’ and reverse primer: 5’-CCAAGATGGGCACCCTCC-3’).
LOX (forward primer: 5’-GGAGCTCAGCATGGTATG-3’ and reverse primer: 5’-CGTGGTCAAGGGTACTTTA-3’). Glyceraldehyde phosphate dehydrogenase (GAPDH) used as an internal control was also amplified using forward primer: 5’-CCTGTCCGGTAGACAAATG-3’, reverse primer: 5’-TGAAGGGTGCTGTAGGC-3’. The cDNA samples were amplified at 95°C for 15 minutes, 93°C for 30 seconds, and 58°C for 30 seconds for a total of 40 cycles. All PCRs were done in an iCycler iQ real-time PCR detection system. The fluorescence threshold values (Ct) were calculated using the manufacturer’s software. Relative mRNA levels were assessed by standardization to GAPDH. Results were expressed as a fold difference in gene expression.

Reverse transcriptase PCR (RT-PCR) was done for COX-2, PGES-2, and LOX, and FLAP using the following conditions. COX-2 denaturation at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute. Oligonucleotide primer sequences used were as follows: 5’-CCTGTCCGGTAGACAAATG-3’, reverse primer: 5’-CGTGGTCAAGGGTACTTTA-3’. PGES-2 denaturation at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, and 72°C for 45 seconds. Oligonucleotide primer sequences used were as follows: 5’-GACCTTACCTTATACAAATACAT-3’, sense primer: 5’-TCTGTGGGTGAACTCTGGCTAG-3’. 5-LOX denaturation was at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 45 seconds. Oligonucleotide primer sequences used were as follows: 5’-GCCGGACTGATGTACCTGTT-3’, reverse primer: 5’-GCTGGGTCAGGGGTACTTTA-3’. FLAP denaturation was at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 50°C for 12 seconds, and 72°C for 1 minute. Oligonucleotide primer sequences used were as follows: 5’-GCTGTCAGAGGGTAGTACCTTA-3’, reverse primer: 5’-GGTGAGCGTCCTTCTCTGTC-3’. The cDNA was denatured at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 52°C for 1 minute. Oligonucleotide primer sequences used were as follows: 5’-GACCTTACCTTATACAAATACAT-3’, sense primer: 5’-GCCGGACTGATGTACCTGTT-3’. LTB4 receptor denaturation was at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 60°C for 20 seconds, and 72°C for 45 seconds. Oligonucleotide primer sequences used were as follows: 5’-TACCCGCAGCTTCAGTACGTTT-3’, reverse primer: 5’-GCCGGACTGATGTACCTGTT-3’. PCR was done using the Taq polymerase master mix (Qiagen Inc.). The PCR products were visualized and photographed under UV illumination.

Inflammatory cytokines and prostaglandin E2 assays

Determination of inflammatory cytokine levels in serum and PGs in SI polyps were evaluated by ELISA–SA Biosciences and Cayman chemical, respectively, as per manufacturers’ instructions. The mouse Inflammatory Cytokines and Chemokines Multi-Analyte ELISA Array Kit analyzes a panel of 12 proinflammatory cytokines using ELISA protocol, all at once under uniform conditions in serum. The cytokines and chemokines represented in this array are interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN-γ, TNF-α, and granulocyte-macrophage colony stimulating factor (GM-CSF). Results are expressed as ng/mL of serum. Determinations were carried out in triplicate from each sample. SI polyps were solubilized with homogenization buffer, and total protein was measured and analyzed for PGE2 content using commercially available ELISA kits (Cayman Chemical). The data were expressed as nanogram of PGE2 produced/mg protein.

Statistical analyses

All results were expressed as means ± SE and statistical significance was analyzed by 1-way ANOVA or Student t test. Differences were considered significant at the P < 0.05 level.

Results

General observations

APCMin/+ mice fed with the control diet showed significantly lower body weight gain than the mice fed with the experimental diets containing either 150 or 300 ppm licofelone (Fig. 1C and D) in both genders. No significant body weight change or noticeable signs of toxicity were observed in treatment groups. The body weight loss in control group mice is mainly due to the increased small intestinal tumor burden and impairment of food absorption and anemia. Our studies have shown that licofelone administered to male wild-type mice (up to 600 ppm in diet) for 6 weeks has not caused any observable toxicity or significant body weight loss (data not shown). Thus, doses applied in the efficacy studies were expected to be nontoxic.

Dietary licofelone dramatically suppresses intestinal polyposis and colon tumors in APCMin/+ mice

APCMin/+ mice spontaneously develop intestinal tumors, mostly in the small intestine with fewer tumors in the colon. All histopathologically classified tumors in the small intestine, as well as those in the colon, were adenomas (adenomatous polyps), with no evidence of local invasion of the lamina propria. Figure 2A and B summarizes the chemopreventive effect of dietary licofelone administered at 150 or 300 ppm on tumor multiplicity in the small intestine. Male and female APCMin/+ mice fed with control diet developed an average of 48.8 ± 4.3 and 34.3 ± 3.8 intestinal polyps, respectively (Fig. 2A and B). Licofelone administration at 150 and 300 ppm for 14 weeks significantly reduced total intestinal tumor multiplicity and size dose dependently in both male and female mice (means ± SEM tumors for 150 ppm: 17 ± 3.7 and 8 ± 1.4, respectively, in male and female mice; and 8.8 ± 1.4 and 5.5 ± 1.8, respectively, in female mice; Fig. 2A and B). Importantly, high-dose licofelone showed approximately 83% (P < 0.0001) intestinal tumor inhibition in both male and female mice. The mean number of colon tumors in male and female mice was 1.83 and 1.0, respectively, in control diet–fed mice; whereas mice fed with 150 ppm licofelone showed colon tumor inhibition of 72% (male) and 67% (female), respectively. It is noteworthy that both male and female mice fed with 300 ppm licofelone showed 100% inhibition of colon tumors (Fig. 2C and D). Interestingly, the number of large-sized polyps (>2 mm) was dramatically reduced with licofelone treatments (Fig. 2E and F). Mice fed with 150 ppm licofelone had 90% (male) and 86% (female) fewer polyps with sizes greater
than 2 mm. Mice fed 300 ppm licofelone showed more than 97% suppression of polyps of greater than 2 mm size in both genders compared with control mice.

MRI of mice for polyp count in small intestine and colon

At the termination of the study, live mice were assessed for total small intestinal polyps and colon tumors by MRI. Images are collected pre- and postinjection of Magnivest and polyps are identified as bright areas accumulating Magnivest (Fig. 1E). This method helps in localizing hidden polyps within the crypts that cannot be evaluated under a stereomicroscope. However, the polyp count was almost identical when compared with that evaluated under dissection microscope after necropsy.

Licofelone decreases serum triglycerides and increases packed cell volume

A decrease in serum TG was observed in mice with fed licofelone compared with control mice. Compared with wild-type C57BL/6J mice, control APCmin/+ mice showed significant increases in serum TGs, whereas TG levels of APCmin/+ mice with fed high-dose licofelone were comparable with those of wild-type mice. Both male and female
mice fed with licofelone diets showed dose-dependent suppression of serum TGs (76.5%, \( P < 0.001 \) or 71.5%, \( P < 0.001 \) with 150 ppm and 82.4% \( P < 0.0001 \) or 79.7% \( P < 0.0001 \) with 300 ppm licofelone, respectively; Fig. 2G and H). Also, we observed a dose-dependent increase in PCV (blood cells) in licofelone-fed male and female APC\(^{Min/+}\) mice (Fig. 2I and J).

Administration of licofelone inhibits intestinal tumor COX and 5-LOX activities

On the basis of radio-HPLC analysis, a significant decrease was seen in total COX and 5-LOX metabolites in intestinal polyps of mice fed with a low-dose licofelone diet. As shown in Fig. 3A–C, mean total PG and thromboxane B2 (TXB2) levels in polyps from control mice versus mice fed with 150 ppm licofelone were 69.2 versus 28.9 pmol/min; PGE2 was 19.3 versus 10.2 pmol/min, and 5-HETE was 26.4 versus 9.3 pmol/min. The total COX and 5-LOX activities were significantly reduced in intestinal polyps from low-dose licofelone-treated mice compared with polyps from control mice by 57% \( (P < 0.001) \) and 64.5% \( (P < 0.0001) \), respectively (Fig. 3A–C). As shown in Fig. 3D, mean total PGE2 levels in SI polyps from control mice versus those from mice fed with 150 ppm licofelone were 355 versus 185 ng/mg protein as measured by ELISA.

Effect of dietary licofelone on intestinal polyp proliferative and apoptotic index

Figure 4A and B summarizes the effects of licofelone on tumor cell proliferation as measured by PCNA overexpression. Qualitative microscopic examination of PCNA-stained sections showed a substantial decrease in PCNA-positive cells in tumors from male mice exposed to licofelone compared with tumors from the mice fed with the control diet. Licofelone at 300 ppm significantly suppressed proliferation in the intestinal polyps as compared with control (Fig. 4A). The quantification of PCNA staining showed 45.1 \pm 4.4 (mean \( \pm \) SEM) PCNA-positive cells in control polyps, as compared with 12.2 \pm 2.4 (mean \( \pm \) SEM) PCNA-positive cells in polyps from licofelone-treated mice, accounting for a decrease in the proliferation index of approximately 70% \( (P < 0.0001) \). Figure 4C and D summarizes the effects of licofelone on tumor cell apoptosis. Qualitative microscopic examination of TUNEL-stained sections showed a substantial increase in TUNEL-positive cells in polyps from mice treated with 300 ppm licofelone. The quantification of tunnel-positive cells in polyps from control–fed mice showed 10.62 \pm 1.34 (mean \( \pm \) SEM), as compared with 51.2 \pm 5.36 (mean \( \pm \) SEM)-positive cells in SI polyps from licofelone-treated mice, accounting for an increase in the apoptotic index by more than 75% \( (P > 0.0001) \). Interestingly, hyperproliferative regions of the small intestines from mice fed 300 ppm licofelone showed a robust increase in apoptosis rather than polyps.

Modulation of COX-2, 5-LOX, PGES-2, FLAP, and LTB4 receptor expressions in SI polyps

Expression levels of COX-2, 5-LOX, PGES-2, FLAP, and LTB4 receptor, which are important AA pathway indicators of proliferation and tumorigenesis, were analyzed in intestinal polyps by IHC, real-time, and RT-PCR (Fig. 5A–J). Dietary administration of 300 ppm licofelone resulted in significant decreases in COX-2 and 5-LOX protein expression in intestinal polyps, as compared with polyps from mice fed with the control diet (Fig. 5A and D). Also, dose-dependent decreases of COX-2, 5-LOX, prostaglandin E synthase-2 (PGES-2), FLAP, and LTB4 receptor mRNA expression levels were observed in tumors from mice fed with licofelone (Fig. 5B, C, and E–J).

Modulation of inflammatory cytokines

Levels of the cytokines IL-1β, IL-2, IL-6, IL-10, IFN-γ, G-CSF, and GM-CSF that either induce COX-2 or act as...
proinflammatory cytokines are significantly decreased in the serum of mice fed with the licofelone diet (Fig. 6). IL-6 was reduced by more than 97% and TNF-α was completely inhibited. More importantly, no significant difference was observed in the expression levels of the anti-inflammatory cytokine IL-4 (Fig. 6).

Discussion

Epidemiologic and clinical studies, as well as experimental colon carcinogenesis, have clearly shown that NSAIDs are effective chemopreventive agents for CRC. NSAIDs and COX-2 selective inhibitors have been tested widely for CRC prevention. However, the gastrointestinal and cardiovascular toxicities exhibited by these agents have prompted the search for novel approaches/agents with similar or higher efficacies, but devoid of unwanted side effects. As rationalized in the introduction, targeting COX and 5-LOX pathways may provide better efficacy without unwanted cardiovascular and gastrointestinal side effects. To date, no systematic studies have been carried out to establish the potential usefulness of dual COX-5-LOX inhibitors for CRC prevention. This study results clearly show that the novel 5-LOX–COX inhibitor licofelone suppresses small intestinal and colon carcinogenesis in both male and female APCMin/+ mice. We showed that licofelone dramatically prevents small intestinal and colon tumor formation in APCMin/+ mice in a dose-dependent manner without any gastrointestinal or other toxicities. A diet with 150 and 300 ppm licofelone resulted in 70% and 100%, respectively, colon tumor suppression in both genders of mice, suggesting the potential usefulness of licofelone as a colon cancer chemopreventive agent.

Intestinal tumor counts and size assessed under dissection microscope were comparable with those analyzed by MRI imaging of live mice. Both methods revealed a remarkable inhibition of polypl size (>2 mm) with 150 ppm licofelone and complete elimination of polyplps of more than 2 mm with 300 ppm licofelone in the feed. The efficacy achieved with licofelone is comparable or more effective than several NSAIDs and COX-2 selective inhibitors, celecoxib and rofecoxib (30–35). For example, in our previous studies, we have shown that 300 ppm celecoxib suppressed small intestinal and colon tumor formation by 69% and 75%, respectively, compared with that in mice fed with control diet. Supplementary data presented in Supplementary Fig. S1, on the effect of 300 ppm of celecoxib in the diet, show inhibition of 66% for formation of SI polyplps and 69% for formation of colonic polyplps in male APCMin/+ mice, similar to our previous reports. Dietary administration of 300 ppm celecoxib suppressed PGE2 levels in intestinal polyplps by 52%, as assessed with radio-HPLC analysis, and by 55% as determined with the EIA method (data not shown). Also, studies by Dr. Dubois’ group (36) showed that a 78% inhibition of small intestinal tumors was associated with a 60% reduction of tumor PGE2 levels in APCMin/+ mice exposed to 1,000 ppm celecoxib in the diet. In comparison, 300 ppm of licofelone suppressed small intestinal and colon tumors by 83% and 100%, respectively, in APCMin/+ mice. Moreover, our results indicate a 55% or 48% reduction in the intestinal tumor PGE2 levels in the mice treated with 150 ppm licofelone as measured by HPLC or ELISA, respectively (Fig 3C and D). Collectively, these results support the better efficacy of licofelone when compared with the COX-2 selective inhibitor celecoxib. Consistent with the current results, we previously reported that...
licofelone administered in male F344 rats inhibits significantly the formation of azoxymethane-induced aberrant colonic crypt foci in a dose-dependent manner (8). In addition, Ye and colleagues (16) showed that the 5-LOX inhibitor AA861 and the COX-2 inhibitor celecoxib combined together provide better efficacy in cigarette smoke–induced colon tumor xenografts. Similar observations were made by Gianchi and colleagues (37) using the 5-LOX inhibitor MK866 in combination with celecoxib in colon tumor xenograft assays. Taken together, these improved efficacy results clearly validate the potential usefulness of licofelone for human colon cancer chemoprevention clinical trials.

The aberrant AA metabolism through COX-2 and its relevance to CRC is well established (38; 39). Increased production of PGE2 was found in the intestinal adenomas of FAP patients and in colon cancer tissues (38). Previous reports suggested that increased PGE2 levels generated by COX-2 and its downstream products, such as PGES-2, 5-LOX, and LTB4R, are involved in colon carcinogenesis. The modulatory effects of licofelone on COX-2 and PGES-2 mRNA expression in intestinal polyps of treated and untreated APCMin/+ mice are shown in Figure 5C. A significant dose-dependent suppression of COX-2 mRNA was observed upon licofelone treatment in APCMin/+ mice. Similar observations were made with the 5-LOX inhibitor MK866 in combination with celecoxib in colon tumor xenografts. Taken together, these improved efficacy results clearly validate the potential usefulness of licofelone for human colon cancer chemoprevention clinical trials.
COX-2 upregulation could accelerate the intestinal polyposis (39). The potential of 5-LOX as a target for colon cancer prevention has been somewhat less extensively studied. However, recent studies have shown the involvement of 5-LOX metabolites, particularly LTs, in inflammation, gastrointestinal ulceration, and colon carcinogenesis (40). In this regard, we have shown previously that chemically induced rat colon tumors abundantly produce 5-LOX metabolites and that suppression of these metabolites by naturally occurring anti-inflammatory agents such as curcumin and caffeic acid esters, in part, is associated with colon tumor inhibition (41, 42). In this study, we found that not only was licofelone able to block the activity of the AA-metabolizing enzymes 5-LOX and COX but also that it inhibited the expression of the 5-LOX–activating protein FLAP and the LTB4 receptor in a dose-dependent manner in intestinal polyps (Fig. 3B and Fig. 5E–G). Previous studies have implicated overexpression of LTB4 receptors in colonic tumors and use of the LTB4 receptor antagonist LY29311 suppressed growth of colon cancer cell lines (15). In the 5-LOX–mediated pathway, 5-HETE is a key intermediate in the generation of colon polyps from untreated mice. Interestingly, hyperproliferation regions of intestinal crypts showed a marked increase in apoptotic cells in the mice fed with licofelone, but not in the intestinal crypts from the mice fed with the control diet. This result suggests that licofelone induces apoptosis at an early stage and inhibits colonocytes in the hyperproliferative regions from transforming further into polyps. The mechanisms through which licofelone inhibits cell proliferation and induces apoptosis have been studied in vitro models (30). However, the exact mechanisms by which licofelone suppresses tumor cell proliferation and induces apoptosis needs further study.

Recent studies suggest that hyperlipidemia may promote colon polyp development in FAP patients and in rodent models of FAP. Thus, antihyperlipidemic agents may be beneficial for colon cancer prevention and treatment (44). Our study shows that licofelone reduced the serum TGs by 71% to 83%, which is very well correlated with polyp inhibition. The TG levels in serum samples from high dose treated mice are comparable with the TG levels in the serum of wild-type mice in both genders.

Licofelone treatment also led to significant decreases in most proinflammatory cytokines (Fig. 6). The levels of circulating IL-6, IL-8, M-CSF, and the IL-1 receptor antagonist significantly increase with the clinical stage of CRC (45, 46). Also, increased levels of IL-6, TNF receptor type I (RI), soluble IL-2 receptor α, and TNF-α were observed with increasing tumor grade and bowel wall invasion (45). In this study, we observed more than 96% decrease in IL-6 and almost complete inhibition of IL-2, IL-12, TNF-α, and G-CSF in licofelone-treated serum samples compared with serum samples from untreated mice (Fig. 6).

Previously, no tumor inhibition studies had been reported in vivo using the dual COX–LOX inhibitor licofelone. Compared with nonsteroidal anti-inflammatory drugs (e.g., celecoxib and sulindac) and other agents studied previously in the APCMin/+ model, the efficacy of licofelone observed in this study is dramatic or comparable or even higher (31–35). Overall, our results show that licofelone, a dual 5-LOX–COX inhibitor, suppresses SI and colonic tumor formation in APCMin/+ mice dose dependently with high efficacy and devoid of unwanted side effects. These findings support further development of licofelone for colon cancer prevention and treatment.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Chemoprevention of Colon and Small Intestinal Tumorigenesis in APC \textsuperscript{Min/+} Mice by Licofelone, a Novel Dual 5-LOX/COX Inhibitor: Potential Implications for Human Colon Cancer Prevention

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