# Cancer Therapy: Preclinical

# Overexpression of 5-Lipoxygenase in Colon Polyps and Cancer and the Effect of 5-LOX Inhibitors *In vitro* and in a Murine Model

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**Abstract**

**Purpose:** Arachidonic acid metabolism via the cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) pathways modulates cell growth and apoptosis. Many studies have examined the effects of COX inhibitors on human colorectal cancer, but the role of 5-LOX in colonic cancer development has not been well studied. The purpose of this study was to evaluate the expression of 5-LOX in colonic polyps and cancer and the effect of 5-LOX inhibition on colon cancer cell proliferation.

**Experimental Design:** Colonic polyps, cancer, and normal mucosa were evaluated for 5-LOX expression by immunohistochemistry. Reverse transcription-PCR was used to establish 5-LOX expression in colon cancer cells. Thymidine incorporation and cell counts were used to determine the effect of the nonspecific LOX inhibitor Nordihydroguaiaretic Acid and the 5-LOX inhibitor Rev5901 on DNA synthesis. A heterotopic xenograft model in athymic mice using HT29 and LoVo human colon cancer cells was used to evaluate the effect of the 5-LOX inhibitor zileuton on tumor growth.

**Results:** 5-LOX is overexpressed in adenomatous polyps and cancer compared with that of normal colonic mucosa. LOX inhibition and 5-LOX inhibition decreased DNA synthesis in a concentration- and time-dependent manner in the Lovo cell line ($P < 0.05$). Inhibition of 5-LOX in an *in vivo* colon cancer xenograft model inhibited tumor growth compared with that of controls ($P < 0.05$).

**Conclusions:** This study showed that 5-LOX is up-regulated in adenomatous colon polyps and cancer compared with normal colonic mucosa. The blockade of 5-LOX inhibits colon cancer cell proliferation both *in vitro* and *in vivo* and may prove a beneficial chemopreventive therapy in colon cancer.

Colorectal cancer is the third most commonly diagnosed cancer and the second most common cause of cancer death in the United States, with ~153,780 new cases and 52,180 deaths expected in 2007 (1). Although there has been significant improvement in screening modalities and early detection, the number of mortalities from colorectal cancer remains substantial. Sequential genetic abnormalities have been characterized in precursor lesions in the colon. A stepwise progression from normal colonic mucosa to colorectal cancer has been mapped out based on accumulated genetic changes (2). Based on this process, the ideal management of this disease would stem from prevention.

Results from epidemiologic studies suggest that a high-fat diet is associated with an increased incidence of tumors at several organ sites including the colon, breast, pancreas, and prostate (3, 4). Cyclooxygenases (COX) are the rate-limiting enzymes in prostaglandin and thromboxane synthesis from arachidonic acid. Early investigations into the role of arachidonic acid metabolism in cancer have focused mainly on the COX pathway, particularly the inducible COX-2 enzyme because of the epidemiologic observation that the incidence of colonic cancer was significantly reduced in regular users of aspirin and other nonsteroidal anti-inflammatory drugs (5). The extracellular stimuli that induce COX-2 include growth factors, cytokines, tumor promoters, hypoxia, radiation, and carcinogens (6). COX-2 has been identified in precursor lesions of several solid tumors (7). Several groups have examined the role of the lipoxygenase (LOX) pathway in carcinogenesis and have shown that both the 5-LOX and 12-LOX pathways play an important role in promoting tumor growth (8–11). We have identified overexpression of the 5-LOX pathway in pancreatic adenocarcinoma tissue compared with normal controls as well as in pancreatic epithelial neoplasias, which are precursor lesions of pancreatic cancer (12). Recently, it was shown that

5-LOX expression is up-regulated in human colorectal cancer specimens, and this correlated with tumor size, depth, and vessel invasion (13). The cancer growth–promoting effects of the downstream products of the 5-LOX pathway such as 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene B4 (LTB4) have also been shown (14). In addition, the efficacy of 5-LOX inhibitors and LTB4 receptor antagonists in cancer cell growth inhibition have been investigated (15–18). Bortuzzo et al. found that LTB4 stimulated proliferation in colon cancer cell lines HT-29 and HCT-15 in a time- and concentration-dependent manner and that LTB4 receptors are expressed in colonic epithelial cells (19). Based on the above evidence, the current study investigated the expression of the 5-LOX pathway in colonic polyps and their progression to cancer. In addition, we hypothesized that the inhibition of 5-LOX in colon cancer cell lines would inhibit cell proliferation both *in vitro* and in a heterotopic xenograft model in athymic mice.

## Materials and Methods

*Immunohistochemistry for 5-lipoxygenase.* Thirty surgical samples of normal colonic mucosa (6), neoplastic colonic polyps (16), and colon cancer (8) specimens were collected and examined at Northwestern Memorial Hospital, Chicago, Illinois. Immunohistochemistry was used to compare 5-LOX expression between adjacent normal colonic mucosa, tubular adenomas, and colon cancer. The monoclonal 5-LOX antibody was purchased from BD Pharmingen. All specimens were fixed in 10% buffered formalin, paraffin embedded, and processed for histology by conventional methods. Sections (4 μm thick) were prepared from paraffin blocks. After deparaffinization, the slides were submerged in methanol containing 0.3% hydrogen peroxide for 30 min at room temperature to inhibit endogenous peroxidase activity. Antigen retrieval for 5-LOX was achieved by incubating the sections in 10 mmol/L citrate buffer (pH 6) in a microwave oven for 12 min (2 min high power, 10 min medium low power). The slides were cooled to room temperature and washed in TBS (0.1 mol/L, pH 7.4). The slides were incubated with normal goat serum for 30 min at room temperature and then with the primary antibody directed against 5-LOX (mouse monoclonal, 1:250 in TBS containing 1% bovine serum albumin) for 18 h at 4°C. The slides were washed again in TBS and incubated with secondary antibody (Multilink) for 10 min at 37°C. Detection of the antibody complex was done by the streptavidin-peroxidase reaction kit. Counterstaining was done with hematoxylin Gill no 2. Staining intensity in epithelial cells was scored as follows: -, no positive staining; +, weakly positive staining; ++, moderately positive staining; and +++, strongly positive staining. The stained tissue samples were scored by a pathologist blinded to the recorded pathology of the slide.

*Cell culture.* The LoVo, HCT116, and HT-29 colon cancer cell lines were purchased from the American Type Culture Collection. Each cell line was grown in DMEM and plated as monolayers in medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% $O_2$ and 5% $CO_2$ at 37°C. The cells were regularly seeded into 75-cm$^2$ flasks with media changes every second or third day. For experiments, cells were grown to 70% confluence, digested with trypsin-EDTA, and plated in 6-, 24-, or 48-well plates.

*Reverse transcription-PCR.* Reverse transcription-PCR was conducted as previously described (20). The 5-LOX primer sequences for quantitative PCR were: sense 5′-CCAGACCATCACCCACCTTC-3′ and antisense 5′-GAATCTCACGTGTGCCACCA-3′. Equal volumes of the PCR product from each sample were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photographed.

*Thymidine incorporation.* Cells were plated in 24-well plates at a concentration of 50,000 cells per well. After reaching 50% confluence, they were incubated in serum-free medium for 24 h, which was then replaced with fresh serum-free medium with or without either

**Table 1.** Expression of 5-LOX in colon samples of 24 patients

| Histology (*n*) | Intensity | | | | Distribution | |
|---|---|---|---|---|---|---|
| | - | + | ++ | +++ | Focal | Diffuse |
| Normal (6) | 6 | 0 | 0 | 0 | 0 | 0 |
| Adenoma (16) | 0 | 5 | 7 | 4 | 13 | 3 |
| Carcinoma (8) | 0 | 0 | 5 | 3 | 1 | 7 |

NOTE: In this collection of tissue samples, some patients contributed samples of more than one type of histology. Six cases contributed samples of normal and adenoma histology, but none contributed more than one tissue sample with the same histology. Staining intensity in epithelial cells was scored as follows: -, no positive staining; +, weakly positive staining; ++, moderately positive staining; +++, strongly positive staining.

nordihydroguaiaretic acid (NDGA; GeroNova Research), a LOX inhibitor (0-100 μmol/L), or Rev5901 (0-15 μmol/L), a specific 5-LOX inhibitor (Calbiochem). After the required period (0-24 h) of culture, cellular DNA synthesis was assayed by adding 0.5 μCi *methyl*-$^3$H thymidine per well and incubating cells for another 6 h. The cells were then washed twice with PBS, fixed with 10% trichloroacetic acid, and solubilized by adding 250 μL of 0.4 mol/L NaOH to each well. Radioactivity, indicating incorporation of *methyl*-$^3$H thymidine into DNA, was measured by adding scintillation cocktail and counting on a scintillation counter (LKB RackBeta; Wallac).

*Heterotopic xenograft tumor model.* Athymic nude mice (BALB/c nu/nu, 5-week old females) were purchased from the Frederick Cancer Research and Development Center. The mice were acclimatized to the animal facility for 1 wk before receiving xenografts. LoVo or HT29 human colon cancer cells ($5 \times 10^6$ cells in 50 μL) were injected into both flanks of 10 and 8 nude mice, respectively. Once visible tumors were established 4 d after injection, the mice were randomized into 2 groups with 4 (HT29) and 5 (LoVo) mice per group. The control groups received daily p.o. dosages of DMSO (0.5 μL/g/d) placed on the back of the tongue; the Zileuton groups received daily p.o dosages of zileuton 100 mg/kg/d in the same volume of DMSO as the controls. Animal weight and tumor size were recorded every third day. The formula for calculating tumor volume was: (length) × (width) × (length+width/2) × 0.526 = volume. After 30 d of treatment, the animals were euthanized and the tumors were carefully dissected and tumor weights measured. This study was approved by the Institutional Animals Care and Use Committee, and all animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals.

*Statistical analysis.* A contingency table $\chi^2$ test was done to analyze the association of 5-LOX immunohistochemical staining intensity with histology in human colon polyp and cancer tissue samples. Data were analyzed by ANOVA with Dunnett's (*in vitro* growth data) or Bonferroni's (*in vivo* tumor size data) post hoc tests for multiple comparisons. This analysis was done with the Prism software package (GraphPad). Data were expressed as mean ± SE. Differences were considered statistically significant with $P < 0.05$.

## Results

*5-LOX expression is up-regulated in adenomatous colonic polyps and in colon cancer.* Using immunohistochemistry, we evaluated the expression of 5-LOX expression in formalin-fixed, paraffin-embedded tissue sections from 6 samples of normal colonic mucosa, 16 tubular adenomas, and 8 colon cancers. Intense positive 5-LOX staining was evident in adenomatous polyps compared with adjacent colonic mucosa from 16 of 16

specimens (Table 1). Normal colonic epithelium was negative for 5-LOX staining aside from the occasional leukocyte in the stroma (Fig. 1A-B). All 16 of the tubular adenomas showed some 5-LOX expression in the nuclear envelope and in the cytoplasm ranging in intensity from 1 to 3 and with 13 of 16 staining in focal areas (Fig. 1C-D; focal areas are magnified). All 8 of the colon cancer tissue specimens analyzed stained positive for 5-LOX with all staining in intensity from 2 to 3 and with a diffuse pattern (Fig. 1E-F). With a contingency table $\chi^2$ test, there was a significant association between staining intensity and histology ($P < 0.0001$) and between staining distribution (focal versus diffuse) and histology ($P < 0.01$). Of note, six of the colon cancers were stage I ($T_1N_0$) and two were stage III ($T_3N_1$). There was no significant relationship between the stage of the colon cancer and the staining intensity or distribution. These results may indicate that 5-LOX expression is activated early in the tumor development process and as the degree of malignancy increases, 5-LOX expression becomes more intense and diffuse in distribution.

*5-LOX is expressed in three different colon cancer cell lines.* Expression of 5-LOX RNA was clearly seen in all three colon cancer cell lines (LoVo, HCT116, and HT29) that were investigated (Fig. 2).

*5-LOX inhibitors decrease proliferation of colon cancer cells in a concentration- and time-dependent manner.* Nonselective LOX blockade with NDGA induced a concentration-dependent inhibition of proliferation (Fig. 3A). In LoVo human colon cancer cells treated with NDGA for 24 hours, thymidine incorporation was significantly decreased [ANOVA, $F = 32$ (6, 53); $P < 0.0001$] to 59.8% at 10 μmol/L ($P < 0.05$), 19.8% at 20 μmol/L, and 1.0% at 100 μmol/L ($P < 0.01$). Nonselective LOX blockade with 10 μmol/L NDGA also caused time-dependent inhibition of proliferation (Fig. 3B). Thymidine incorporation was significantly decreased [ANOVA, $F = 46$ (3, 11); $P < 0.0001$] to 78.9% at 6 hours, 58.7% at 12 hours, and 44.8% at 24 hours (all $P < 0.01$). Specific 5-LOX blockade with Rev5901 for 24 hours also induced a concentration-dependent inhibition of proliferation (Fig. 3C). In LoVo human colon cancer cells treated with Rev5901, thymidine incorporation was significantly decreased [ANOVA, $F = 205$ (5, 102); $P < 0.0001$] to 50.7% at 3.75 μmol/L, 16.7% at 7.5 μmol/L, 11.7% at 15 μmol/L, and 8.4% at 30 μmol/L (all $P < 0.01$). Specific 5-LOX blockade with Rev5901 induced a time-dependent decrease in proliferation (Fig. 3D). In LoVo human colon cancer cells treated with 15 μmol/L Rev5901, thymidine incorporation was significantly decreased [ANOVA,
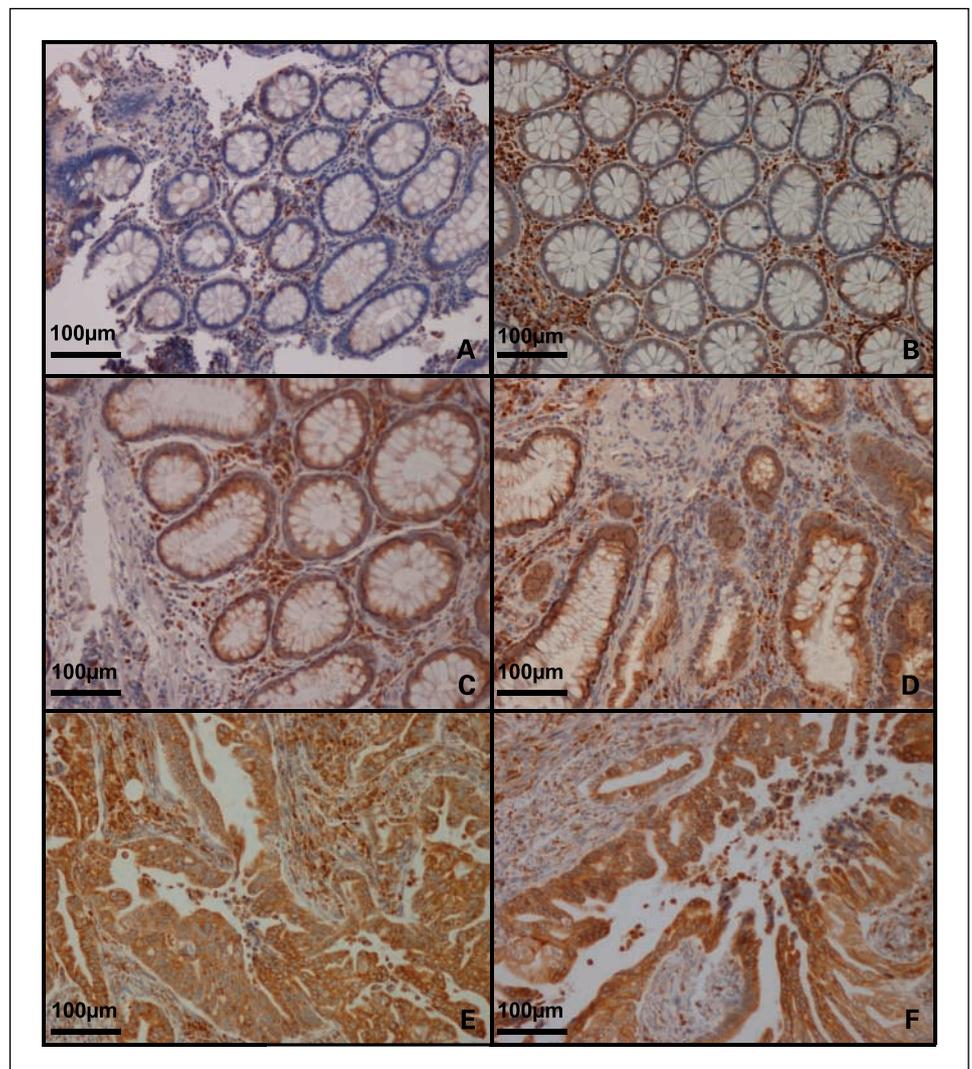


**Fig. 1.** Immunohistochemical analysis of 5-LOX expression in normal colonic mucosa (*A, B*), tubular adenomas (*C, D*), and cancers (*E, F*). Tubular adenomas and cancer show progressively more intense and diffuse nuclear and cytoplasmic staining for 5-LOX (*brown*) compared with adjacent normal colonic mucosa. Leukocytes in the stroma also stain for 5-LOX. Original magnification, ×20.
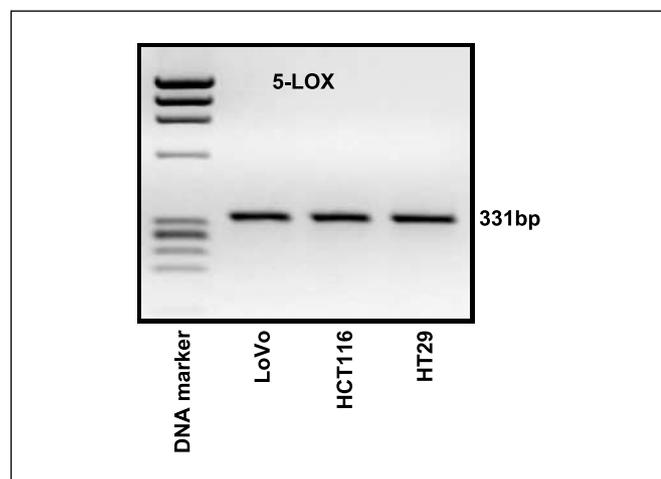
**Fig. 2.** Ethidium bromide – stained agarose gel showing the products of 5-LOX reverse transcription-PCR in three colonic cancer cell lines (LoVo, HCT116, and HT29). The single band for each cell line indicates that 5-LOX mRNA is expressed in all three cell lines.

$F = 35$ (3, 60); $P = 0.003$] to 45.4% of control at 12 hours and to 9.3% of control at 24 hours ($P < 0.01$).

***5-LOX inhibition inhibits growth of colon cancer xenografts in athymic mice.*** Zileuton inhibited the growth of both human LoVo and HT29 colon cancer xenografts in athymic mice, measured both as tumor volume over time and tumor weight at the end of the experiment (Fig. 4A-D). Measurement of LoVo tumor volumes over time revealed significant differences between control and treated animals for the first time on day 15, and this difference was maintained until the end of the experiment (Fig. 4A; $P < 0.001$). At the end of the experiment,

the weights of the LoVo tumors were significantly lower in the zileuton-treated animals compared with the controls (Fig. 4C; $P < 0.01$). The tumor volumes of the HT29 colon cancer xenografts also revealed significant differences between control and treated animals over time with the first at day 19, and this persisted till the end of the experiment (Fig. 4B; $P < 0.0001$). Evaluation of the HT29 tumors revealed a similar pattern of results with the tumor weights of the zileuton-treated animals significantly less than the controls at the end of the experiment (Fig. 4D; $P = 0.024$).

## Discussion

Animal experiments have shown that polyunsaturated fats containing linoleic acid, the precursor of arachidonic acid, play an important role in cancer development and growth (3 – 8). Although the role of the COX pathway in colonic cancer development has been widely studied, investigation of the role of the LOX pathway in this cancer has been limited. 5-LOX overexpression has been found in several human cancers including prostate, pancreatic, colon, bladder, esophageal, and testicular cancer (12, 21 – 25). In previous studies we have shown that both 5-LOX and 12-LOX mRNA and protein are expressed in human pancreatic cancer cell lines but not in normal human pancreatic ductal cells (17). We have also shown that 5-LOX is overexpressed early in pancreatic intra-epithelial neoplasms, the precursor lesions of pancreatic cancer, as well as in pancreatic cancer tissues (16, 17). Two studies have shown a relationship between 5-LOX overexpression in colon cancer and poor prognosis (13). Several studies have shown that eicosanoids produced by 5-LOX play important roles in the development of cancer (26). The inhibition of cancer cell growth and the induction of apoptosis by 5-LOX inhibitors or
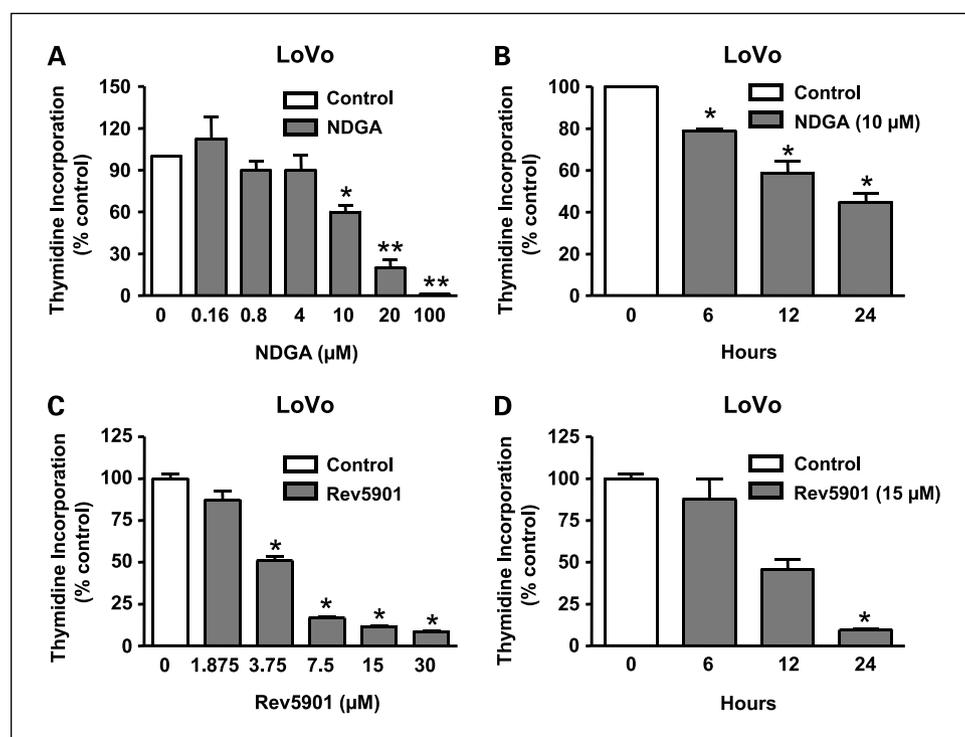


**Fig. 3.** Effect of lipoxygenase inhibitors on growth of LoVo human colonic cancer cells. The nonselective LOX inhibitor NDGA inhibited cell proliferation by decreasing $^3$H-thymidine incorporation in a (*A*) concentration-dependent and (*B*) time-dependent manner. The selective 5-LOX inhibitor REV5901 inhibited cell proliferation by decreasing $^3$H-thymidine incorporation in a (*C*) concentration-dependent and (*D*) time-dependent manner. Results are expressed as % of control of thymidine incorporation and represent mean $\pm$ SE of four separate experiments. *, $P < 0.05$; **, $P < 0.01$ compared with control.

**Fig. 4.** Effect of the selective 5-LOX inhibitor zileuton (100 mg/kg/d) on growth of human colonic cancer zenografts of LoVo (*A* and *C*) and HT29 (*B* and *D*) cells in athymic mice. Tumor burden was measured by both volume (*A* and *B*) throughout the experiment and by tumor weight (*C* and *D*) at the end of the experiment. Zileuton significantly decreased the size of tumors in both groups. *, $P < 0.05$; **, $P < 0.01$.
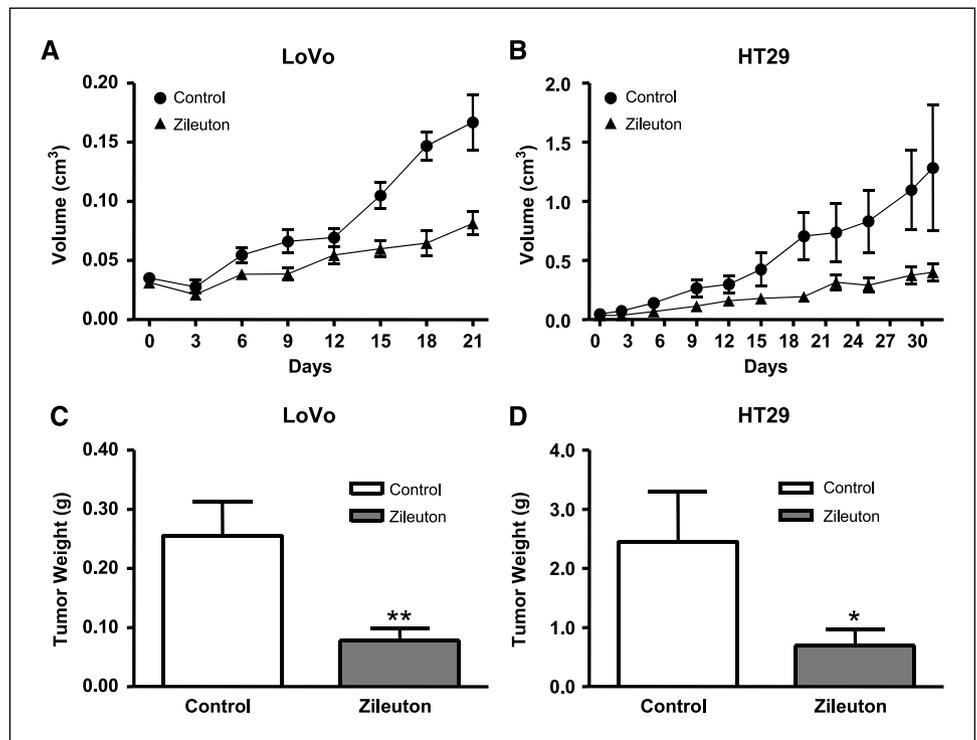
downstream LTB4 inhibitors have been shown in renal, esophageal, pancreas, prostate, and oral cancers (25 – 33).

In the present study, we investigated the expression of 5-LOX in normal mucosa, tubular adenomas, and colon cancer specimens. In addition, we evaluated the effects of both specific and nonspecific inhibitors of 5-LOX on colon cancer cell proliferation *in vitro* and on a heterotopic xenograft model in athymic mice *in vivo*. We have shown that 5-LOX is progressively overexpressed in human tubular adenomas of the colon and colon cancer specimens, whereas it is only seen in occasional cells of normal colonic mucosa. These results indicate that as lesions acquire a more malignant phenotype, the expression of 5-LOX increases.

There is some evidence that adverse bronchial and nasal reactions to aspirin and other COX inhibitors are associated with the production of downstream products of 5-LOX. We do not have any evidence if any of our patients were chronic aspirin users. However, immunohistochemical studies in bronchial biopsies of patients with aspirin-intolerant asthma showed an increased expression of leukotriene C4 synthase but no increased expression of 5-LOX. In nasal polyps from similar patients an increased expression of 5-LOX was seen, but this was in the eosinophils and not the epithelium (34, 35).

Because adenomatous colonic polyps are the accepted precursors of invasive carcinoma of the colon, 5-LOX inhibitors may turn out to be effective chemopreventive agents to stop the progression from polyps to invasive colon cancers. To evaluate the effect of both nonspecific and specific 5-LOX inhibitors on colon cancer cell proliferation, we first established that 5-LOX was expressed in three well-established colon cancer cell lines (LoVo, HCT116, and HT29). We were then able to show that in the LoVo and HT29 cell lines both the nonselective 5-LOX inhibitor NDGA and the 5-LOX specific inhibitor REV5901 were able to inhibit proliferation in both a time- and concentration-dependent fashion. To evaluate the potential of 5-LOX inhibition *in vivo*, we examined the effects of the 5-LOX inhibitor zileuton in athymic mice bearing s.c. transplanted human colon cancer cells. The heterotopic xenograft tumor model is widely used and accepted as an effective method for studying the *in vivo* effects of anticancer treatments. Zileuton was chosen for these studies because it is the only clinically available 5-LOX inhibitor (33). There have been several studies showing the chemopreventive effects of zileuton in carcinogenesis models in lung, esophageal, and oral cancers (25, 36 – 38). Li et al. and Sun et al. both showed that zileuton prevented oral carcinogenesis at the postinitiation stage in a 7,12-dimethylbenz(*a*)anthracene – induced hamster model (38). Oral administration of zileuton markedly inhibited tumor growth (both LoVo and HT29 cells) compared with that in vehicle-treated control animals during the treatment period. There did not seem to be any toxic effects of zileuton at the dose used in these animals. These results indicate that 5-LOX inhibition may be a valuable addition to the drugs used for the treatment colon cancer. Further studies are needed to evaluate the efficacy and safety of 5-LOX inhibition both in animal models and in human trials.

In conclusion, these studies show that 5-LOX is up-regulated in colon polyps and colon cancer compared with normal colonic mucosa, indicating that 5-LOX expression is an early indicator of malignant progression. Furthermore, 5-LOX inhibition reduces colonic cancer proliferation both *in vitro* and *in vivo*. These findings provide evidence that 5-LOX plays a role in colon cancer development and may be a target for chemoprevention of colon cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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