Chronic use of aspirin and related drugs to reduce cancer risk is limited by unwanted side effects. Thus, we assessed the efficacy associated with different dosing regimens of aspirin and naproxen. Azoxymethane (AOM)-rat colon cancer model was used to establish the pharmacodynamic efficacy of aspirin and naproxen under different dosing regimens. Colon tumors were induced in rats (36/group) by two weekly doses of AOM. At the early adenoma stage, rats were fed diets containing aspirin (700 and 1,400 ppm) or naproxen (200 and 400 ppm), either continuously, 1 week on/1 week off, or 3 weeks on/3 weeks off, or aspirin (2,800 ppm) 3 weeks on/3 weeks off. All rats were euthanized 48 weeks after AOM treatment and assessed for efficacy and biomarkers in tumor tissues. Administration of aspirin and naproxen produced no overt toxicities. Administration of different treatment regimens of both agents had significant inhibitory effects with clear dose-response effects. Aspirin suppressed colon adenocarcinoma multiplicity (both invasive and noninvasive) by 41% ($P < 0.003$) to 72% ($P < 0.0001$) and invasive colon adenocarcinomas by 67%–91% ($P < 0.0001$), depending on the treatment regimen. Naproxen doses of 200 and 400 ppm inhibited invasive adenocarcinoma multiplicity by 53%–88% ($P < 0.0001$), depending on the dosing regimen. Colonic tumor biomarker analysis revealed that proliferation (proliferating cell nuclear antigen and p21), apoptosis (p53 and Caspase-3), and proinflammatory mediators (IL1β and prostaglandin E2) were significantly correlated with the tumor inhibitory effects of aspirin and naproxen. Overall, our results suggest that intermittent dosing regimens with aspirin or naproxen demonstrated significant efficacy on the progression of adenomas to adenocarcinomas, without gastrointestinal toxicities.
COX-2 inhibitors (17–19). Meta-analyses of >50 randomized trials and systematic reviews suggest that regular use of aspirin for at least 3 years is associated with a decreased incidence of colonic adenomas, colorectal cancer, metastatic colorectal cancer, and deaths due to colorectal cancer (19). In other studies, individuals 50–69 years of age considered at high risk for colorectal cancer took daily low-dose aspirin for >5-years to reduce their risk for colorectal cancer, according to a set of recommendations from the U.S. Preventive Services Task Force (20).

Numerous studies have shown that nonaspirin NSAIDs may also have a role in preventing colorectal cancer because long-term use of aspirin and other NSAIDs is associated with gastrointestinal (GI) toxicities. In particular, individuals >65 years of age or older may not benefit from long-term NSAID use due to the risks for GI bleeding, stroke, and other side effects (21, 22). COX-2 inhibitors, particularly celecoxib, have been studied to overcome the GI toxicities. Mechanistically, these inhibitors are more efficacious than aspirin and/or other NSAIDs and were proven to be effective in both preclinical and randomized clinical trials (18, 23–25). However, some of beneficial effects of celecoxib have been compromised by the increased risk of cardiovascular fatalities with study subjects who had a previous history of atherosclerotic heart disease (18, 26–27). This information suggests an interaction of celecoxib in subjects with high risk of cardiovascular events (18).

Notably, a study published in the Lancet (14) supported the usefulness of aspirin for prevention of colorectal and other cancers. This randomized trial, involving over 25,000 people, compared daily use of aspirin with no aspirin and showed that long-term (20-year) use of aspirin decreased the risk for colorectal cancer by 40%. Remarkably, aspirin use for 5 years or more reduced the risk of proximal colon cancer by about 70% (P < 0.0001). This finding is especially significant because of the association of serrated polyps in the proximal colon with colorectal cancer, and the difficulty in diagnosing these proximal polyps through colonoscopy screening.

With the exception of aspirin, various NSAIDs have been implicated in human cardiovascular risk. The cardiovascular risk with COX-2–selective inhibitors has often been highlighted; however, somewhat less discussed is the risk associated with other NSAIDs, such as diclofenac, piroxicam, ibuprofen, and naproxen. A recent systematic review included about 30 case-controlled studies involving 184,946 cardiovascular events and described outcomes in >2.7 million exposed individuals with relative risk (RR) estimates for major cardiovascular events associated with use of individual NSAIDs reported for different doses and in populations with low and high background risks for cardiovascular events (28). These results suggest that the highest overall risks were seen with rofecoxib (RR = 1.45; 95% confidence interval (CI), 1.33–1.59) and diclofenac (RR = 1.40; 95% CI, 1.27–1.55). The lowest risks were with ibuprofen (RR = 1.18; 95% CI, 1.11–1.25), and naproxen (RR = 1.09; CI, 1.02–1.16). Notably, naproxen was risk-neutral at all doses, and had a significantly lower risk than did ibuprofen (RR = 0.92; 95% CI, 0.87–0.99). Overall, existing data suggest that, among widely used nonaspirin NSAIDs, naproxen is least likely to increase cardiovascular risk. Previous preclinical studies showed that naproxen's chemopreventive efficacy was superior to that of several NSAIDs in a rat colon cancer model (29).

It is clear that further development strategies that would reduce the side effects but maintain the efficacy of aspirin and naproxen for high-risk cohorts of colorectal cancer is an important issue. Recent studies in the bladder cancer model by Lubet and colleagues (30) showed that intermittent dosing with the NSAID naproxen, which should reduce gastric toxicity, retained preventive activity. In this study, we examined both aspirin and naproxen in the colon cancer model to determine whether we could reproduce this prior observation. Toward this end, we have designed experiments using different dosing regimens of human equivalent doses (HED) of aspirin and naproxen to assess their efficacy on inhibiting adenoma progression to colon carcinoma formation in a well-established rat model of AOM-induced colorectal cancer (Supplementary Table S1B). We specifically tested the chemopreventive efficacy of 700 and 1,400 ppm dietary aspirin or 200 and 400 ppm naproxen dosing either continuously, 1 week on/1 week off, or 3 weeks on/3 weeks off to evaluate whether intermittent dosing regimens demonstrate significant efficacy without GI toxicities. In addition, we evaluated the efficacy of a single dose of aspirin (2,800 ppm) administered 3 weeks on/3 weeks off on inhibition of the progression of colonic adenoma to adenocarcinoma.

Materials and Methods
Chemicals, animals, and diets
Aspirin and azoxymethane were procured from Sigma-Aldrich. Naproxen was obtained from Medisca. Five-week-old pathogen-free inbred male Fischer (F344) rats were obtained from Envigo Animal Resources. Animals were housed in ventilated cages under standardized conditions (21°C, 50% humidity. 12 hour-light/12 hour-dark cycle, 20 air changes per hour) in the University of Oklahoma Health Sciences Center rodent barrier facility. All animal studies have been conducted in accordance with, and with the approval of Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center (Oklahoma City, OK). Semipurified modified American Institute of Nutrition modified (AIN)-76A diet ingredients were purchased from Bio-Serv. Diet ingredients containing casein, 20%; corn starch, 52%; dextrose, 13%; corn oil, 5.0%; alphacel/cellulose, 5.0%; DL-methionine, 0.3%; mineral mix AIN, 3.5%; vitamin mix, AIN, 1.0%; and...
choline bitartrate, 0.2% were mixed thoroughly so that all of the ingredients were uniformly distributed in the diet (23). Both control and experimental diets were prepared weekly and stored in a cold room. Rats were allowed ad libitum access to the respective diets and to automated tap water purified by reverse osmosis. Food cups were replenished with fresh diet 3 times weekly.

**Dose selection of aspirin and naproxen**

For the male F344 rats (~330 g weight), the HEDs of aspirin and naproxen were approximately 3,000 ppm and approximately 1,000 ppm, respectively, in the diet based on adjustments to metabolic rates (Supplementary Table S1B). To select proper dosing regimens for efficacy studies, a 6-week chronic dosing of select doses were assessed in the male F344 rats. Briefly, at 7 weeks of age, rats in each group (6 rats) were fed control and experimental diets containing aspirin (500, 1,000, 1,500, 2,000, and 3,000 ppm) or naproxen (125, 250, 500, 750, and 1,000 ppm) until termination of the study, that is, after 6 weeks on experimental diets. Body weights and symptoms of toxicity were recorded once weekly for 6 weeks. At termination, rats were assessed for gross pathologic observations, including gastrointestinal toxicities and key serum liver enzyme levels.

**Efficacy bioassay: determination of the efficacy of different dosing regimens of aspirin and naproxen on colon adenocarcinoma formation**

The experimental design to determine the efficacy of different dosing regimens of aspirin and naproxen is shown in Fig. 1A. A total of 594 rats were used in the efficacy study (Supplementary Tables S3 and S4). At 8 weeks of age, male F344 rats (control: 66 AOM-treated; 24 vehicle (saline)-treated; drug treatment groups: 36 AOM-treated/group, 6 vehicle (saline)-treated) on modified AIN-76A control diet were injected with AOM (15 mg/Kg body weight)/saline s.c. once a week for 2 weeks (Fig. 1A) beginning at 8 weeks of age. Five weeks after AOM treatment, rats were fed with experimental diets containing aspirin at 700 and 1,400 ppm and naproxen at 200 and 400 ppm (continuously or intermittent dosing: 1 week on/1 week off; or 3 weeks on/3 weeks off) and a single dose of 2,800 ppm aspirin intermittent dosing (3 weeks on/3 weeks off). Animals were maintained on control or experimental diets until the termination of the experiment. Body weights were recorded every week for the first 10 weeks, and then every 2 weeks. Animals were monitored daily for general health. The experiment was terminated 48 weeks after the second AOM treatment, at which time all animals were euthanized via CO2 euthanasia. Blood was collected from each animal to assess liver and hematopoietic toxicities. At termination, rats were assessed for gross pathologic observations, including GI toxicities. After laparotomy, the entire small intestine and large intestine were resected and opened longitudinally, and the contents were flushed with normal saline. Colon and small intestinal tumors were noted grossly for their location, number, and size (Fig. 1B).

**Histopathology**

For histopathologic evaluation of tumors, intestines were fixed in 10% neutral buffered formalin, processed and embedded in paraffin blocks, cut into multiple sections, and placed on slides for hematoxylin and eosin (H&E) staining. The slides stained with the standard H & E protocol were examined by a pathologist, who was blinded to the treatment groups. The histologic criteria used for the classification of intestinal tumors were described previously (31, 32). The end points used were incidence and multiplicity of tumors. At the end of the study, more than 92% of the rats fed control diet developed colon tumors. All colonic tumors were classified as adenomas and adenocarcinomas (invasive and noninvasive; ref. 31). The invasive adenocarcinomas invaded the muscularis mucosa deep into the intestinal wall and beyond. The noninvasive adenocarcinomas were those growing outward toward the intestinal lumen but not invading the muscularis mucosa. These tumors were usually well-differentiated adenocarcinomas (Fig. 1B and C).

**Liver and hematopoietic toxicities**

Sera samples from each treatment group were analyzed for key liver enzyme profiles of ALKP, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using the IDEXX Catalyst Dx system. For hematopoietic toxicities, whole blood was collected in vet-collect tubes with or without heparin/EDTA anticoagulant and analyzed by IDEXX ProCyte Dx to compare various hematologic parameters, including percentage hematocrit, content of hemoglobin, and counts for neutrophils, lymphocytes, and platelets.

**IHC**

Embedded colonic tumor tissues (noninvasive adenocarcinomas) from rats treated with different regimens were assessed for expression levels of proliferating cell nuclear antigen (PCNA), p21, p53, and Caspase-3 by IHC as per previously published methods (33). Nonimmune rabbit IgGs were substituted for primary antibodies as negative controls. IHC specimens were observed using an Olympus microscope IX71. Digital computer images were recorded with an Olympus DP70 camera.

**IHC scoring**

Scoring was done according to the intensity of the nucleic or cytoplasmic staining (no staining = 0, weak staining = 1, moderate staining = 2, and strong staining = 3) and prevalence of stained cells (1%–10% = 1, 11%–20% = 2, 21%–50% = 3, and >50% = 4), as established...
previously (34). The final immunoreactive score was determined by multiplying the intensity scores by the prevalence of positivity scores of stained cells, yielding a minimum score of 0 and a maximum score of 12. Tumor cells with a brown intensity were considered positive. Scoring was performed by two investigators blinded to the identity of the PCNA-positive cells at 400× magnification. The PCNA-positive proliferation index (PI) was determined by dividing the number of positive cells by the number of negative cells and multiplying by 100.

Cytokines and prostaglandin E2 assays

Determination of key inflammatory cytokines and arachidonic acid pathway metabolite prostaglandin E2 (PGE2) levels in colonic tumors were evaluated by Cytokine Array MER-004A (ELISA - Qiagen) and PGE2 ELISA Kit (Cayman Chemical), respectively, as per the manufacturer’s instructions and previously published methods (35). The rat cytokines array includes IL1β, IL6, IL10, and IL12. The rationale for the selection of these cytokines was based on previous colon cancer studies, which demonstrated either significant upregulation or downregulation (35). Colonic tumors were solubilized with homogenization buffer, and total protein was measured. Results are expressed as pg/mg of protein. Determinations were carried out in triplicate from each sample.

Statistical analysis

Mean body weight gain and markers are presented (mean ± SEM) and were compared among the rats fed control diet and rats fed different dietary dosing regimens of the test agents. ANOVA or t test was used to compare the effects of various dosing regimens of aspirin and naproxen on body weights. Tumor incidence (percentage of rats with colonic tumors) among the rats receiving control diet and different dietary groups was analyzed using the two-tailed Fisher exact test. Tumor multiplicity (total number of tumors per animal) was presented as Mean ± SEM and significance of the difference between the different means among various groups was calculated using the unpaired t test with Welch correction. The data were analyzed using GraphPad Prism 7.01 software.

Results

Dose selection

As shown in Supplementary Tables S1 and S2, aspirin up to 2,000 ppm had no effect on body weight retardation,
external signs of toxicity, and key liver enzyme indicators of toxicity. At 3,000 ppm, chronic feeding of aspirin for 6 weeks produced significant ($P < 0.0012$; $\approx 7.7\%$) body weight loss without any gross changes in major organs (lungs, heart, liver, spleen, kidneys, pancreas, etc.), but with an increase in liver AST and ALT levels in the serum samples and grade 1 stomach ulceration as determined by histology (Supplementary Tables S1 and S2). Animals fed naproxen up to 500 ppm showed no body weight loss compared with animals fed control diet. However, rats given 750 ppm naproxen showed a significant ($P < 0.0001$) retardation of body weight gain within 1 week of exposure; however, no rats died after 6 weeks of chronic administration. Rats fed 1,000 ppm naproxen showed significant toxicity, and most died within the 3 weeks of treatment due to GI toxicity. On the basis of this 6-week chronic feeding study, we selected doses of 700 and 1,400 ppm of aspirin and 200 and 400 ppm naproxen for the continuous and intermittent dosing regimens, as well as 2,800 ppm aspirin for the 3 weeks on/3 weeks off regimen.

**Efficacy bioassay/general observations**

Rats were monitored for body weight gain or retardation from 7 weeks of age until the termination of the experiment, that is, 58 weeks of age. As anticipated, vehicle-treated control rats had higher body weight gain than did AOM-treated rats. Administration of aspirin (700, 1,400, or 2,800 ppm) and naproxen (200 or 400 ppm) did not produce any body weight loss and/or any external signs of toxicities throughout the experimental period (Supplementary Tables S3 and S4). Neither aspirin nor naproxen, given in different doses and regimens, produced any stomach ulcerations (Supplementary Tables S5 and S6) or any other gross organ toxicities. Furthermore, serum levels of ALKP, LDH, AST, and ALT were in the normal range in the rats exposed to various dosing regimens of aspirin and naproxen at the termination (Supplementary Tables S5 and S6).

**Histopathology and AOM-induced colonic tumor incidence and multiplicity in rats fed control diet**

Histopathologically, colonic tumors were either adenomas or adenocarcinomas (malignant), which were further classified as noninvasive and invasive adenocarcinomas, as described above. Noninvasive adenocarcinomas are predominantly exophytic and tubular, whereas invasive carcinomas are both endophytic and exophytic, and mostly mucinous/signet-ring type (Fig. 1C). Because test agents were applied at the late aberrant crypt foci (ACF)/early adenoma stage, this histologic classification provides a better understanding of the effect of aspirin and naproxen dosing regimens on progression of ACF/adenoma to malignant carcinomas. In vehicle-treated rats fed control or experimental diets, no colonic tumors were observed. In the rats fed control diet and treated with AOM, >92% rats developed colonic tumors (adenomas and adenocarcinomas). A total of 48% of these rats developed adenomas, and >86% developed adenocarcinomas (Fig. 2). With regard to colon tumor multiplicity (Fig. 3), rats fed control diet and treated with carcinogen showed 0.75 ± 0.10 (mean ± SEM, $N = 66$) colon adenomas and 2.44 ± 0.10 (mean ± SEM, $N = 66$) colon adenocarcinomas, of which about 39% were highly invasive adenocarcinomas, while the remaining tumors were malignant noninvasive adenocarcinomas, as described above.

**Efficacy of aspirin on adenocarcinoma progression**

Figures 2 and 3 show the effect of aspirin on AOM-induced colon adenoma and adenocarcinoma incidence and multiplicities. Aspirin had no statistically significant effect on the incidence of colonic adenomas with any dose or treatment regimen. Aspirin had a significant inhibitory effect ($P < 0.012–0.0001$) on adenocarcinoma incidence in a dose-responsive manner (Fig. 2). Treatment with low dose aspirin inhibited adenocarcinoma incidence (35.4%–38.9%) and multiplicity (41%–47.9%) except for the 3 weeks on/3 weeks off regimen, which had no

![Figure 2](attachment:figure2.png)

*Figure 2.*

Effect of different doses and dosing regimens of aspirin (continuous, 700 and 1,400 ppm; 1 week on/1 week off, 700 and 1,400 ppm; and 3 weeks on/3 weeks off, 700, 1,400, and 2,800 ppm) on AOM-induced adenoma incidence (A); noninvasive adenocarcinoma incidence (B); and invasive carcinoma incidence (C). Values are Mean ± SEM. Control group, $n = 66$; treatment groups, $n = 36$. Differences between control and treatment groups are significant per two-tailed Fisher exact test.
significant effect on the incidence of adenocarcinomas but did show significant reductions in multiplicity (44.3%; \( P < 0.0002 \)). Similarly, all of the treatment regimes with higher doses of aspirin at 1,400 ppm (\( P < 0.012–0.0001 \)) and 2,800 ppm (\( P < 0.0001 \)) suppressed the incidence of invasive adenocarcinomas (Fig. 2) whereas only the continuous treatment regimes decreased the incidence of the noninvasive adenocarcinomas. Irrespective of doses and/or treatment regimes, aspirin suppressed invasive adenocarcinomas of the colon by 59%–85% (\( P < 0.0002–0.0001 \)). These results are highly significant, given that the applied dose levels represent 350 mg aspirin/day, which is equal to approximately 700 ppm (mg) aspirin in diet.

With regard to the multiplicity of colon adenocarcinoma, aspirin showed a significant inhibitory effect with different treatment regimes in a dose-response manner (Fig. 3). Taken together, total adenocarcinomas (both invasive and noninvasive) multiplicities were suppressed by 41% (\( P < 0.003 \)) to 72% (\( P < 0.0001 \)). Aspirin suppressed invasive colon adenocarcinomas by 67% (\( P < 0.0001 \)) to 91% (\( P < 0.0001 \)) with different treatment regimes. Overall, the use of low dose aspirin for 3 weeks on/3 weeks off significantly protected against AOM-induced colonic adenocarcinoma, particularly invasive adenocarcinomas.

**Effect of naproxen on AOM-induced colonic tumor incidence and multiplicity**

Figures 4 and 5 summarize the effect of naproxen on AOM-induced colonic adenoma and adenocarcinoma incidence and multiplicities. Naproxen showed a modest (13.2%–19.4%; \( P > 0.05 \)) to significant (32.4%–42.2%; \( P < 0.003–0.0001 \)) inhibitory effect on adenocarcinoma incidence in different treatment regimes. As compared with continuous or one 1 on/1 week off exposure to low dose naproxen (200 ppm), the 3 weeks on/3 weeks off treatment exhibited a diminished inhibitory effect [19.4%–35.4% (\( P = 0.065–0.0013 \)) vs. 13.2% (\( P = 0.18 \))] on colon adenocarcinoma incidence. A similar treatment regimen with higher doses of naproxen (400 ppm) suppressed the total adenocarcinoma incidence (32.4%; \( P < 0.003 \)). Similar to aspirin, naproxen doses and/or dosing regimens greatly suppressed incidence of invasive adenocarcinoma of the colon up to 79% (\( P < 0.0001 \)). With regard to colon adenocarcinoma multiplicity, naproxen showed a significant inhibitory effect with different treatment regimes that suggest a dose-response effect (Fig. 5). Naproxen different dosing regimes significantly suppressed noninvasive adenocarcinoma by 44.7%–61.3% (\( P < 0.0006–<0.0001 \)) with exception of low dose naproxen 3 weeks on/3 weeks off regimen, which lacks the protective effect. Taken together, total adenocarcinomas (both invasive and noninvasive) multiplicities were suppressed 23% (\( P < 0.03 \)) to 71% (\( P < 0.0001 \)), with a trend of high dose showing greater inhibition, irrespective of treatment regimen. Naproxen also suppressed invasive colon adenocarcinomas by 53% (\( P < 0.0009 \)) to 88% (\( P < 0.0001 \)) with different treatment regimes.

**Effect of aspirin and naproxen on tumor cell proliferation and apoptosis markers**

Tables 1 and 2 summarize the effects of different dosing regimes of aspirin and naproxen, respectively, on markers of proliferation, apoptosis, and inflammation in colonic tumors. Dietary administration of aspirin, 700 and 1,400 ppm, either continuously (\( P < 0.006–0.0003 \)) or 1 week on/1 week off (\( P < 0.023–0.0015 \)), significantly inhibited the colon tumor PI (PCNA) in a dose-dependent manner, compared with the colonic tumor PI in rats fed control diet (Table 1). Aspirin 1,400 ppm (\( P < 0.005 \)) and 2,800 ppm (\( P < 0.0016 \)) administered 3 weeks on/3 weeks off significantly suppressed colon tumor PI; however, no such effect was seen with lower dose aspirin delivered in a similar dosing regimen. Similarly, different dosing regimes of naproxen showed significant (\( P < 0.0035–0.0001 \)) suppression of PI, with the exception of the low...
dose naproxen 3 weeks on/3 weeks off dose regimen, compared with control diet (Table 2). However, the p21 expression level in colonic tumors was significantly increased ($P < 0.006$–$0.0001$) in rats fed aspirin and various dosing regimens of naproxen compared with expression in rats fed control diet. p53 and caspase-3 colonic tumor expression levels were high in various dosing regimens of aspirin and naproxen treatments compared with control diet. An increased correlation trend was observed between p53/caspase-3 expression levels in continuous dosing and higher intermittent dosing regiments of aspirin and naproxen.

**Effect of aspirin and naproxen on colonic tumor inflammatory markers**

As shown in Tables 1 and 2, colonic tumor proinflammatory cytokines IL1β, IL6, and IL12 were significantly ($P < 0.009$–$0.0001$) inhibited by all dosing regimens of aspirin and naproxen, with the exception of a modest reduction of IL12 ($P = 0.08$) in the colonic tumors of rats fed 700 ppm aspirin for a 3 weeks on/3 weeks off regimen, compared with colonic tumors of rats fed control diet. In contrast, IL10 levels were significantly increased in the colonic tumors of rats fed different dosing regimens of aspirin ($P < 0.043$–$0.0001$) and naproxen ($P < 0.004$–$0.0001$) compared with tumors of rats fed control diet. PGE$_2$, a metabolite of COX-1/COX-2 and the direct target of aspirin and naproxen, was analyzed to determine the effectiveness of different dosing regimens. Colonic tumor PGE$_2$ levels were significantly inhibited in rats fed different dosing regimens of aspirin (23.3%–67.8%; $P < 0.0002$–$0.0001$) and naproxen (27.9%–69.4%; $P < 0.0001$) compared with levels in tumors from rats fed control diet. A trend of dosing regimen effects of aspirin and naproxen was observed in the inhibition of PGE$_2$ levels.

**Discussion**

This is, to our knowledge, the first report using different dosing regimens of aspirin and naproxen in a well-established model of colorectal cancer. Our laboratory and others have extensively studied the chemopreventive effects
Table 1. Effect of aspirin dosing regimens on the modulation of markers of proliferation, apoptosis, and inflammation in AOM-induced rat colonic adenocarcinoma

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Frequency of dose administration</th>
<th>PCNA PI (1–9)</th>
<th>p21 expression IHC score (1–12)</th>
<th>p53 expression IHC score (1–12)</th>
<th>Caspase-3 expression IHC score (1–12)</th>
<th>Inflammatory cytokine levels (pg/mg protein) mean ± SEM, N = 6–9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control diet</td>
<td>–</td>
<td>56.7 ± 5.2</td>
<td>0.5 ± 0.2</td>
<td>2.1 ± 0.3</td>
<td>3.1 ± 0.5</td>
<td>2,673 ± 95 557 ± 36 273 ± 18 42 ± 4.7</td>
</tr>
<tr>
<td>2 Aspirin</td>
<td>Continuous</td>
<td>38.5 ± 3.7</td>
<td>4.7 ± 0.9</td>
<td>3.8 ± 0.6</td>
<td>6.5 ± 1.1</td>
<td>1,556 ± 58 322 ± 23 185 ± 14 69 ± 5.8</td>
</tr>
<tr>
<td>3 Aspirin</td>
<td>1,400 ppm</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.04</td>
<td>P &lt; 0.008</td>
<td>1,163 ± 42 247 ± 17 134 ± 11 84 ± 7.1</td>
</tr>
<tr>
<td>4 Aspirin</td>
<td>1 week on/1 week off</td>
<td>42.3 ± 4.1</td>
<td>3.6 ± 0.8</td>
<td>3.9 ± 0.7</td>
<td>5.9 ± 0.9</td>
<td>1,673 ± 67 356 ± 29 202 ± 16 62 ± 5.2</td>
</tr>
<tr>
<td>5 Aspirin</td>
<td>1 week on/1 week off</td>
<td>33.7 ± 2.8</td>
<td>6.9 ± 1.2</td>
<td>5.3 ± 1.0</td>
<td>7.7 ± 1.1</td>
<td>1,338 ± 48 298 ± 23 168 ± 9.8 73 ± 6.1</td>
</tr>
<tr>
<td>6 Aspirin</td>
<td>1,400 ppm</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.005</td>
<td>2,152 ± 63 429 ± 27 234 ± 18 54 ± 4.2</td>
</tr>
<tr>
<td>7 Aspirin</td>
<td>700 ppm</td>
<td>P &lt; 0.023</td>
<td>P &lt; 0.023</td>
<td>P &lt; 0.02</td>
<td>P &lt; 0.01</td>
<td>1,572 ± 85 311 ± 22 172 ± 13 74 ± 6.3</td>
</tr>
<tr>
<td>8 Aspirin</td>
<td>3 weeks on/3 weeks off</td>
<td>47.3 ± 4.8</td>
<td>2.9 ± 0.9</td>
<td>1.8 ± 0.9</td>
<td>4.2 ± 0.8</td>
<td>1,987 ± 49 311 ± 22 172 ± 13 74 ± 6.3</td>
</tr>
<tr>
<td>9 Aspirin</td>
<td>2,800 ppm</td>
<td>P = 0.1</td>
<td>P = 0.06</td>
<td>P = 0.38</td>
<td>P = 0.13</td>
<td>2,152 ± 63 429 ± 27 234 ± 18 54 ± 4.2</td>
</tr>
<tr>
<td>10 Aspirin</td>
<td>3 weeks on/3 weeks off</td>
<td>37.7 ± 3.5</td>
<td>3.5 ± 0.7</td>
<td>2.8 ± 0.6</td>
<td>4.5 ± 0.7</td>
<td>1,858 ± 66 377 ± 21 200 ± 12 60 ± 4.7</td>
</tr>
<tr>
<td>11 Aspirin</td>
<td>200 ppm</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.15</td>
<td>P &lt; 0.063</td>
<td>2,357 ± 65 429 ± 27 234 ± 18 54 ± 4.2</td>
</tr>
<tr>
<td>12 Aspirin</td>
<td>400 ppm</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.009</td>
<td>P &lt; 0.011</td>
<td>2,357 ± 65 429 ± 27 234 ± 18 54 ± 4.2</td>
</tr>
<tr>
<td>13 Aspirin</td>
<td>1 week on/1 week off</td>
<td>34.7 ± 3.8</td>
<td>4.7 ± 0.8</td>
<td>4.2 ± 0.8</td>
<td>6.1 ± 0.9</td>
<td>1,574 ± 46 342 ± 26 187 ± 14 79 ± 6.4</td>
</tr>
<tr>
<td>14 Aspirin</td>
<td>200 ppm</td>
<td>P &lt; 0.003</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.009</td>
<td>P &lt; 0.006</td>
<td>1,108 ± 51 223 ± 19 137 ± 11 93 ± 7.1</td>
</tr>
<tr>
<td>15 Aspirin</td>
<td>400 ppm</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.003</td>
<td>P &lt; 0.003</td>
<td>1,574 ± 46 342 ± 26 187 ± 14 79 ± 6.4</td>
</tr>
<tr>
<td>16 Aspirin</td>
<td>3 weeks on/3 weeks off</td>
<td>45.3 ± 4.7</td>
<td>2.6 ± 0.6</td>
<td>2.9 ± 0.7</td>
<td>3.7 ± 0.6</td>
<td>2,019 ± 67 408 ± 33 203 ± 16 64 ± 5.3</td>
</tr>
<tr>
<td>17 Aspirin</td>
<td>200 ppm</td>
<td>P = 0.07</td>
<td>P &lt; 0.001</td>
<td>P = 0.14</td>
<td>P &lt; 0.24</td>
<td>1,647 ± 55 291 ± 17 172 ± 13 82 ± 6.7</td>
</tr>
<tr>
<td>18 Aspirin</td>
<td>400 ppm</td>
<td>P &lt; 0.003</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.011</td>
<td>1,647 ± 55 291 ± 17 172 ± 13 82 ± 6.7</td>
</tr>
</tbody>
</table>

NOTE: Mean ± SEM (N = 6–9). Values are significantly different from control diet group per t test with Welch correction.

Table 2. Effect of naproxen dosing regimens on the modulation of markers of proliferation, apoptosis, and inflammation in AOM-induced rat colonic adenocarcinoma

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Frequency of dose administration</th>
<th>PCNA PI (1–9)</th>
<th>p21 expression IHC score (1–12)</th>
<th>p53 expression IHC score (1–12)</th>
<th>Caspase-3 expression IHC score (1–12)</th>
<th>Inflammatory cytokine levels (pg/mg protein) mean ± SEM, N = 6–9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control Diet</td>
<td>–</td>
<td>56.7 ± 5.2</td>
<td>0.5 ± 0.2</td>
<td>2.1 ± 0.3</td>
<td>3.1 ± 0.5</td>
<td>2,673 ± 95 557 ± 36 273 ± 18 42 ± 4.7</td>
</tr>
<tr>
<td>2 Naproxen</td>
<td>Continuous</td>
<td>32.3 ± 3.4</td>
<td>5.3 ± 0.61</td>
<td>4.4 ± 0.9</td>
<td>5.8 ± 1.0</td>
<td>1,458 ± 47 288 ± 21 168 ± 13 75 ± 6.7</td>
</tr>
<tr>
<td>3 Naproxen</td>
<td>400 ppm</td>
<td>P &lt; 0.008</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.011</td>
<td>P &lt; 0.001</td>
<td>943 ± 38 192 ± 14 97 ± 8.9 108 ± 8.2</td>
</tr>
<tr>
<td>4 Naproxen</td>
<td>1 week on/1 week off</td>
<td>22.4 ± 2.3</td>
<td>8.9 ± 1.3</td>
<td>5.2 ± 0.83</td>
<td>8.8 ± 14</td>
<td>1,574 ± 46 342 ± 26 187 ± 14 79 ± 6.4</td>
</tr>
<tr>
<td>5 Naproxen</td>
<td>200 ppm</td>
<td>P &lt; 0.003</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.009</td>
<td>P &lt; 0.006</td>
<td>1,108 ± 51 223 ± 19 137 ± 11 93 ± 7.1</td>
</tr>
<tr>
<td>6 Naproxen</td>
<td>400 ppm</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.003</td>
<td>P &lt; 0.003</td>
<td>1,574 ± 46 342 ± 26 187 ± 14 79 ± 6.4</td>
</tr>
<tr>
<td>7 Naproxen</td>
<td>3 weeks on/3 weeks off</td>
<td>45.3 ± 4.7</td>
<td>2.6 ± 0.6</td>
<td>2.9 ± 0.7</td>
<td>3.7 ± 0.6</td>
<td>2,019 ± 67 408 ± 33 203 ± 16 64 ± 5.3</td>
</tr>
<tr>
<td>8 Naproxen</td>
<td>200 ppm</td>
<td>P = 0.07</td>
<td>P &lt; 0.001</td>
<td>P = 0.14</td>
<td>P &lt; 0.24</td>
<td>1,647 ± 55 291 ± 17 172 ± 13 82 ± 6.7</td>
</tr>
<tr>
<td>9 Naproxen</td>
<td>400 ppm</td>
<td>P &lt; 0.003</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.005</td>
<td>P &lt; 0.011</td>
<td>1,647 ± 55 291 ± 17 172 ± 13 82 ± 6.7</td>
</tr>
</tbody>
</table>

NOTE: Mean ± SEM (N = 6–9). Values are significantly different from control diet group per t test with Welch correction.
of both aspirin and a number of NSAIDs (6–10, 30, 36). Dose optimization and tolerability results indicate that rat tolerable doses of aspirin and naproxen, when administered through the diet, are <3,000 ppm and <750 ppm, respectively. These dose-tolerability studies in F344 rats provided essential information on different dose selection and dosing regimens of aspirin and naproxen for the efficacy studies and may assist in making informed dose selections for future studies. The purpose of this study was to investigate whether intermittent dosing regimens are effective in preventing the progression of colon ACF adenoma to adenocarcinomas. Furthermore, assessment of markers of colonic tumor cell proliferation, apoptosis, and inflammation provided valuable insights about the different dosing regimens and dose-response effects.

This study extends our previous work, which identified that aspirin and naproxen show colon cancer chemopreventive effects when administered continuously (8, 29, 37). The results from this research revealed that intermittent dosing regimens of aspirin and naproxen significantly inhibited colonic ACF adenoma progression to adenocarcinoma when assessing tumor multiplicity and further showed a trend toward a dose-dependent effect. Lower doses of aspirin and naproxen administered on a schedule of 3 weeks on/3 weeks off showed significant inhibition of colon adenocarcinoma multiplicity, although to a lesser degree than observed with other dosing regimens. Different dosing regimens, including 3 weeks on/3 weeks off, of both aspirin and naproxen significantly inhibited invasive adenocarcinoma multiplicity, suggesting potential antimetastatic effects.

Aspirin had no significant effects of colonic adenoma formation, suggesting that treatment with aspirin has little effect on the early stages of colonic tumor development during the ACF to adenoma transition (Fig. 2). This is not surprising as the study was designed to begin treatment during the early stages when ACF and early adenomas may have already formed, and thus mimics the likely clinical scenario encountered in a prevention setting in the human population. Although treatment with naproxen exhibited no statistically significant effects on adenoma incidence, different dosing regimens of naproxen inhibited colonic adenoma multiplicity by 12%–64% ($P = 0.3$–$<0.0001$), suggesting better efficacy than aspirin in inhibiting ACF progression to adenoma. Aspirin and naproxen dosing regimens significantly inhibited noninvasive adenocarcinoma multiplicity which was dependent on the agent, dose, and dosing frequency (Figs. 3 and 5). Overall, the results observed with the effects of these agents on noninvasive adenocarcinoma tumor multiplicity suggest that lower doses and a lower frequency of administration had only modest inhibitory effects. Importantly, our observations showed that different dosing regimens of both aspirin and naproxen were most effective at inhibiting more advanced invasive adenocarcinomas by 67%–91% ($P < 0.0001$) and 53.2%–88.3% ($P < 0.001$–$<0.0001$), respectively.

These results clearly suggest that both aspirin and naproxen are effective in blocking colonic invasive carcinoma growth, even with lower doses and less frequency of exposure. These observations on the potent inhibitory effects of aspirin and naproxen further validate our previous work with aspirin at lower continuous dosing regimens through dietary administration in the rat-AOM model, in which noninvasive adenocarcinomas were modestly inhibited compared with the significant inhibition of invasive carcinoma (8). In addition, a number of clinical observations support the notion that aspirin/NSAIDs use improves disease-free and overall survival of patients with metastatic colorectal cancer (38–40). Recently, Zhao and colleagues showed that patients with cancer using NSAIDs had significantly reduced risk of metastasis development of several cancers, regardless of prediagnostic or postdiagnostic use (41). Researchers have also shown that use of aspirin post diagnosis improves survival of patients with colon cancer (42). Taken together, these observations on invasive carcinoma inhibition show that low dose and less frequent use of aspirin and naproxen has chemopreventive potential for patients with high risk colon cancer.

PCNA expression levels in colonic tumor cells act as indicators of PI (43). Our results showed that colonic tumor PI was significantly reduced after all dosing regimens of aspirin and naproxen, except for the low dose/low frequency exposure groups, compared with levels in tumors from rats fed control diet. Aspirin and naproxen showed dose-dependent inhibition of tumor cell proliferation. These findings support our previous reports that NSAIDs suppress tumor growth by blocking cell proliferation (29, 43). Both aspirin and naproxen significantly induced another established marker of tumor cell inhibition, p21waf1/cip, in colonic tumors (29). Previously, we reported that NSAIDs and several other chemopreventive agents that inhibit colon tumor growth are associated with p21waf1/cip induction (29, 35, 43). Levels of p53 expression, which regulates both proliferation and apoptosis and caspase-3 expression, an indicator of apoptosis, were significantly induced with continuous and 1 week on/1 week off dosing regimens of aspirin and naproxen, indicating colonic tumor cell apoptosis as part of tumor cell elimination. The multiple mechanisms by which aspirin and NSAIDs suppress tumor cell proliferation and apoptosis have been extensively studied (7, 44).

It has been well-established that proinflammatory cytokines in the tumor microenvironment significantly aid the aggressive tumor growth by immune evasion mechanisms. In this study, ILβ, IL6, and IL12 proinflammatory cytokine levels were significantly reduced in the colonic tumors of rats fed different dosing regimens of NSAIDs compared with the colonic tumors of rats fed control diet. Consistent with these results, previous reports from our laboratory
and others have shown that NSAIDs significantly inhibit the circulating and colonic tumor proinflammatory cytokines (29, 35). IL1β, IL6, and IL12 levels were upregulated in blood and colonic tumors of patients with cancer and have been implicated as major factors in tumor progression and poor prognosis (45). Several studies have reported that high IL1β concentrations within the tumor microenvironment are associated with a more aggressive tumor phenotype (45). The exact mechanisms by which these proinflammatory cytokines promote tumor growth remain unclear, although a wide range of mechanisms are implicated (45). IL1β has been shown to increase the Wnt/β-catenin signaling in promoting colon tumor growth (46). In another study, IL1β was shown to increase stemness and invasiveness in colon cancer (47). Studies have also shown that IL6 released by colon tumor-associated fibroblasts is critical for tumor angiogenesis, and application of anti-IL6 receptor antibody suppressed angiogenesis and inhibited colonic tumor cell–stroma interactions (45).

The role of IL12 and IL10 as tumor promoters and inhibitors is somewhat controversial. Previously, we showed that colonic tumors and serum levels of IL12 were high in control diet–fed Apcmin/+ mice compared with mice fed diet containing the NSAID lidofoleone (35). Our present findings on inhibition of IL12 levels by aspirin and naproxen are in-line with previous publications. Some studies show that IL10 serum levels increase over time during colorectal cancer progression, suggesting a tumor-promoting role of IL10 in patients with colorectal cancer (48, 49), whereas IL10 appears to play a protective role in animal models of colorectal cancer. IL10 was required for regulatory T cells (Treg) to reduce tumor burden in Apcmin/+ mice (49). Moreover, recent studies show that oral administration of IL10 microparticles decreased polyposis in the Apcmin/+ model by suppressing the development of IL17-producing Tregs (49). Thus, these findings on the induction of IL10 by aspirin and naproxen further support the antitumor effects of IL10. However, the exact mechanisms by which IL10 induction by NSAIDs in AOM-induced rat colonic tumors occur need further investigation.

The role of PGE2 is extensively studied in colon cancer and forms the critical basis for the development of NSAIDs as chemopreventive drugs for colon cancer prevention (7, 22–24). Over 3 decades, our laboratory and others have shown that overexpression of COX-1 and, particularly, COX-2 lead to high levels of PGE2 in clinical samples from colonic tumors and colonic tumors from animal models (10, 22, 23, 29, 35). Thus, it is logical that a significant inhibition of colonic tumor PGE2 levels could be achieved from different dose regimens of aspirin and naproxen, compared with those levels in colonic tumors from rats fed control diet. PGE2 has been shown to promote colon tumors by increasing cell proliferation, invasiveness, and stemness (22). Taken together, our results show the profound inhibitory effects of different dosing regimens of aspirin and naproxen on AOM-induced colonic invasive adenocarcinoma reflected by decreases in PGE2, IL1β, and IL6, which are implicated in tumor cell invasiveness and stemness (49).

In summary, these results suggest that the dosing regimen of 1 week on/1 week off provides significant protective effects on colon adenocarcinoma inhibition, similar to continuous dosing. A particularly interesting observation is that AOM-induced invasive adenocarcinomas were profoundly inhibited by even low dose and low frequency (3 weeks on/3 weeks off) regimens of aspirin and naproxen. Inhibition of PCNA, proinflammatory cytokines, and PGE2, and induction of p21, p53, caspase-3, and IL10 significantly correlated with colon tumor inhibition induced by aspirin and naproxen. Overall, our preclinical findings show that use of an intermittent dosing regimen for aspirin and other NSAIDs in patients with high risk colon cancer might be a beneficial approach that appears to maintain chemopreventive efficacy and while having the potential to reduce side effects associated with long term NSAID treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Interruption Dosing Regimens of Aspirin and Naproxen Inhibit Azoxymethane-Induced Colon Adenoma Progression to Adenocarcinoma and Invasive Carcinoma

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