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Intermittent dosing regimens of aspirin and naproxen inhibit azoxymethane-induced rat colon adenoma progression to adenocarcinoma and invasive carcinoma

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Abstract

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, one week on/one week off, or three weeks on/three weeks off, or aspirin (2,800 ppm) three weeks on/three 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 non-invasive) 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 (PCNA, p21), apoptosis (p53, Caspase-3), and proinflammatory mediators (IL-1 β , PGE₂) 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 GI toxicities.

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Keywords

Colorectal cancer; aspirin; naproxen; colonic polyps; anti-inflammatory drugs

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Introduction

Colorectal cancer (CRC) is a major public health issue worldwide, with an estimated 861,000 deaths and 1.8 million new cases annually (1). Despite proven prevention strategies, CRC remains the second leading cause of cancer-related mortality in the United States, accounting for >50,000 deaths (2). Although there have been significant advances in CRC management strategies, the 5-year survival of patients with Stage IV cancers remains poor (<10%). More than 20% of those CRC patients who have surgical resections are prone to recurrence within one year (3-5). Among the chemopreventive agents for CRC, aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) were shown to be beneficial. Over the past three decades, a compelling body of research has shown that NSAIDs inhibit colorectal carcinogenesis in preclinical models (6-11), clinical observations, and randomized clinical trials (12-17). The CRC-preventive effects of NSAIDs are diverse, based on epidemiologic data and intervention trials in patients with familial polyposis and randomized controlled trials of aspirin and selective COX-2 inhibitors (17-19). Meta-analyses of >50 randomized trials and systematic reviews suggest that regular use of aspirin for at least three 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 to 69 years of age considered at high-risk for CRC took daily low-dose aspirin for >5-years to reduce their risk for CRC, according to a set of recommendations from the US Preventive Services Task Force (20).

Numerous studies have shown that non-aspirin NSAIDs may also have a role in preventing CRC since long-term use of aspirin and other NSAIDs is associated with 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 CRC by 40%. Remarkably, aspirin use for five 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 CRC, and the difficulty in diagnosing these proximal polyps through colonoscopy screening.

With the exception of aspirin, various NSAIDs have been implicated in human cardiovascular (CV) risk. The CV 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 thirty case-controlled studies involving 184,946 CV events and described outcomes in >2.7 million exposed individuals with relative risk (RR) estimates for major CV events associated with use of individual NSAIDs reported for different doses and in populations with low and high background risks for CV events (28). These results suggest that the highest overall risks were seen with rofecoxib (RR = 1.45; 95% 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; 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; CI: 0.87, 0.99). Overall, existing data suggest that, among widely used non-aspirin NSAIDs, naproxen is least likely to increase CV 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 CRC is an important issue. Recent studies in the bladder cancer model by Lubet *et al.* (30) showed that intermittent dosing with the NSAID naproxen, which should reduce gastric toxicity, retained preventive activity. In the present study we examined both aspirin and naproxen in the colon cancer model to determine whether we could reproduce this prior observation. Towards this end, we have designed experiments using different dosing regimens of human equivalent doses (HEDs) 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 CRC (Supplementary Table 1b). We specifically tested the chemopreventive efficacy of 700 ppm and 1,400 ppm dietary aspirin or 200 ppm and 400 ppm naproxen dosing either continuously, one week on/one week off, or three weeks on/ three weeks off to evaluate if 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 three weeks on/ three weeks off on inhibition of the progression of colonic adenoma to adenocarcinoma.

Material and Methods:

Chemicals, animals, and diets:

Aspirin and azoxymethane were procured from Sigma-Aldrich (St. Louis, MO). Naproxen was obtained from Medisca (Irving TX). 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, 12h-light/12h-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 (IACUC) at the University of Oklahoma Health Sciences Center. Semi-purified modified American Institute of Nutrition modified (AIN)-76A diet ingredients were purchased from Bioserv (Flemington, NJ). 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 three times weekly.

Dose-selection of aspirin and naproxen.

For the male F344 rats (~330 gram weight), the human equivalent doses (HEDs) of aspirin and naproxen were ~3,000 ppm and ~1,000 ppm, respectively, in the diet based on adjustments to metabolic rates (Supplementary Table 1b). To select proper dosing regimens for efficacy studies, a six-week chronic dosing of select doses were assessed in the male F344 rats. Briefly, at seven weeks of age, rats in each group (6 rats) were fed control and experimental diets containing aspirin (500 ppm, 1,000 ppm, 1500 ppm, 2,000 ppm, and 3,000 ppm) or naproxen (125 ppm, 250 ppm, 500 ppm, 750 ppm, and 1,000 ppm) until termination of the study, i.e., after six weeks on experimental diets. Body weights and symptoms of toxicity were recorded once weekly for six weeks. At termination, rats were assessed for gross pathological 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 Figure 1a. A total of 594 rats were used in the efficacy study (Supplemental Table 3 and 4). At eight 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 two weeks (Fig. 1a) beginning at eight 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: one week on/one week off; or three weeks on/ three weeks off) and a single dose of 2,800 ppm aspirin intermittent dosing (three weeks on/ three 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 two 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 pathological observations, including gastrointestinal 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 (Figure 1b).

Histopathology.

For histopathological 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 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 histological 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 non-invasive) (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 (Figure 1b and 1c).

Liver and hematopoietic toxicities.

Sera samples from each treatment group were analyzed for key liver enzyme profiles of ALKP, LDH, ALT, and 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 hematological parameters, including percentage hematocrit, content of hemoglobin, and counts for neutrophils, lymphocytes, and platelets.

Immunohistochemistry (IHC).

Embedded colonic tumor tissues (non-invasive adenocarcinomas) from rats treated with different regimens were assessed for expression levels of PCNA, p21, p53, and Caspase-3 by IHC as per previously published methods (33). Nonimmune rabbit Igs 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, strong staining = 3) and prevalence of stained cells (1–10% = 1, 11–20% = 2, 21–50% = 3, and >50% = 4), as previously established (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 (PGE₂) assays.

Determination of key inflammatory cytokines and arachidonic acid pathway metabolite PGE₂ levels in colonic tumors were evaluated by ELISA - Qiagen (cytokine array MER-004A) and Cayman Chemical (PGE₂ ELISA kit), respectively, as per the manufacturers' instructions and previously published methods (35). The rat cytokines array includes IL-1 β , IL-6, IL-10, and IL-12. The rationale for the selection of these cytokines was based on previous colon cancer studies, which demonstrated either significant up-regulation or down regulation (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 $\pm$ 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's Exact* test. Tumor multiplicity (total number of tumors per animal) was presented as Mean $\pm$ SEM and significance of the difference between the different means among various groups was calculated using the unpaired *t*-test with Welch's correction. The data were analyzed using GraphPad Prism 7.01 software.

Results

Dose selection.

As shown in Supplemental Tables 1 and 2, 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 six weeks produced significant ($p < 0.0012$; ~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 (Suppl. Tables 1 and 2). 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 one week of exposure; however, no rats died after six weeks of chronic administration. Rats fed 1,000 ppm naproxen showed significant toxicity, and most died within the three weeks of treatment due to gastrointestinal toxicity. Based on this six-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 three weeks on/three weeks off regimen.

Efficacy bioassay/general observations.

Rats were monitored for body weight gain or retardation from seven weeks of age until the termination of the experiment, i.e., 58 weeks of age. As anticipated, vehicle-treated control rats had higher body weight gain than did AOM-treated rats. Administration of aspirin (700 ppm, 1,400 ppm, or 2,800 ppm) and naproxen (200 ppm or 400 ppm) did not produce any body weight loss and/or any external signs of toxicities throughout the experimental period

(Suppl. Table 3 & 4). Neither aspirin nor naproxen, given in different doses and regimens, produced any stomach ulcerations (Suppl. Table 5 & 6) 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 (Suppl. Table 5 & 6). .

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 non-invasive and invasive adenocarcinomas, as described above. Non-invasive adenocarcinomas are predominantly exophytic and tubular, whereas invasive carcinomas are both endophytic and exophytic, and mostly mucinous/signet-ring type (Figure 1c). Since 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). Forty-eight percent of these rats developed adenomas, and >86% developed adenocarcinomas (Figure 2). With regard to colon tumor multiplicity (Figure 3), rats fed control diet and treated with carcinogen showed 0.75 ± 0.10 (mean $\pm$ SEM, $N=66$) colon adenomas and 2.44 ± 0.10 (mean $\pm$ SEM, $N=66$) colon adenocarcinomas, of which about 39% were highly invasive adenocarcinomas, while the remaining tumors were malignant non-invasive adenocarcinomas, as described above.

Efficacy of aspirin on adenocarcinoma progression:

Figures 2 and 3 show the effect of aspirin on AOM-induced colonic 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 ($p<0.012$ to 0.0001) on adenocarcinoma incidence in a dose-responsive manner (Figure 2). Treatment with low dose aspirin inhibited adenocarcinoma incidence (35.4% - 38.9%) and multiplicity (41% - 47.9%) except for the three weeks on/three weeks off regimen, which had no significant effect on the incidence of adenocarcinomas but did show significant reductions in multiplicity (44.3%, $p<0.0002$). Similarly, all of the treatment regimens with higher doses of aspirin at 1,400 ppm ($p<0.012$ - $p<0.0001$) and 2,800 ppm ($p<0.0001$) suppressed the incidence of invasive adenocarcinomas (Figure 2) whereas only the continuous treatment regimens decreased the incidence of the non-invasive adenocarcinomas. Irrespective of doses and/or treatment regimens, aspirin suppressed invasive adenocarcinomas of the colon by 59% to 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 ~700 ppm (mg) aspirin in diet.

With regard to the multiplicity of colon adenocarcinoma, aspirin showed a significant inhibitory effect with different treatment regimens in a dose-response manner (Figure 3). Taken together, total adenocarcinomas (both invasive and non-invasive) 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 regimens. Overall, the use of low-dose aspirin for three weeks on/three 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%, $p<0.003$ - $p<0.0001$) inhibitory effect on adenocarcinoma incidence in different treatment regimens. As compared with continuous or one week on/one week off exposure to low-dose naproxen (200 ppm), the three weeks on/three weeks off treatment exhibited a diminished inhibitory effect (19.4%-35.4% [$p=0.065$ - $p<0.0013$] 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 regimens that suggest a dose-response effects (Figure 5). Naproxen different dosing regimens significantly suppressed non-invasive adenocarcinoma by 44.7%-61.3% ($p<0.0006$ - $p<0.0001$) with exception of low-dose naproxen three weeks on/three weeks off regimen, which lacks the protective effect. Taken together, total adenocarcinomas (both invasive and non-invasive) 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 regimens.

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

Table 1 and Table 2 summarize the effects of different dosing regimens 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 one week on/one week off ($p<0.023$ - 0.0015), significantly inhibited the colon tumor proliferation index (PI) (PCNA) in a dose-dependent manner, compared with the colonic tumor PI-Index in rats fed control diet (Table 1). Aspirin 1,400 ppm ($p<0.005$) and 2,800 ppm ($p<0.0016$) administered three weeks on/three weeks off significantly suppressed colon tumor PI-Index; however, no such effect was seen with lower-dose aspirin delivered in a similar dosing regimen. Similarly, different dosing regimens of naproxen showed significant ($p<0.0035$ - <0.0001) suppression of PI-Index, with the exception of the low-dose naproxen three weeks on/3 weeks off dose regimen, compared with control diet (Table 2). However, the p21 expression level in colonic tumors was significantly increased ($p<0.016$ - 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 regimens of aspirin and naproxen.

Effect of aspirin and naproxen on colonic tumor inflammatory markers:

As shown in Table 1 and Table 2, colonic tumor proinflammatory cytokines IL-1 β , IL-6, and IL-12 were significantly ($p<0.009$ - 0.0001) inhibited by all dosing regimens of aspirin and naproxen, with the exception of a modest reduction of IL-12 ($p=0.08$) in the colonic tumors of rats fed 700 ppm aspirin for a three weeks on/3 weeks off regimen, compared with colonic tumors of rats fed control diet. In contrast, IL-10 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. PGE₂, 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 PGE₂ 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 PGE₂ 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 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 the present study was to investigate whether intermittent dosing regimens are effective in preventing the progression of colonic 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.

The present study extends our previous work, which identified that aspirin and naproxen show colon cancer chemopreventive effects when administered continuously (8, 37, 38). The results from the current 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 three weeks on/three weeks off showed significant inhibition of colon adenocarcinoma multiplicity, although to a lesser degree than observed with other dosing regimens. Different dosing regimens, including three weeks on/3 weeks off, of both aspirin and naproxen significantly inhibited invasive adenocarcinoma multiplicity, suggesting potential anti-metastatic 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 (Figure 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% ($p=0.3$ - $p<0.0001$), suggesting better efficacy than aspirin in inhibiting ACF progression to adenoma. Aspirin and naproxen dosing regimens significantly inhibited non-invasive adenocarcinoma multiplicity which was dependent on the agent, dose, and dosing frequency (Figures 3 and 5). Overall, the results observed with on the effects of these agents on non-invasive 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% ($p<0.001$ - $p<0.0001$), 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 non-invasive 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 (39-41). Recently, Zhao et al. showed that cancer patients using NSAIDs had significantly reduced risk of metastasis development of several cancers, regardless of pre-diagnostic or post-diagnostic use (42). Researchers have also shown that use of aspirin post diagnosis improves survival of patients with colon cancer (43). 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 proliferative index (PI) (44). 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, 44). Both aspirin and naproxen significantly induced another established marker of tumor cell inhibition, p21^{waf1/cip}, in colonic tumors (29). Previously, we reported that NSAIDs and several other chemopreventive agents that inhibit colon tumor growth are associated with p21^{waf1/cip} induction (29, 35, 44). Levels of p53 expression, which regulates both proliferation and apoptosis, and caspase-3 expression, an indicator of apoptosis, were significantly induced with continuous and one week on/one 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, 45).

It has been well established that proinflammatory cytokines in the tumor microenvironment significantly aid the aggressive tumor growth by immune evasion mechanisms. In the

present study, IL- β , IL-6, and IL-12 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). IL- β , IL-6, and IL-12 levels were upregulated in blood and colonic tumors of cancer patients and have been implicated as major factors in tumor progression and poor prognosis (46). Several studies have reported that high IL-1 β concentrations within the tumor microenvironment are associated with a more aggressive tumor phenotype (46). The exact mechanisms by which these proinflammatory cytokines promote tumor growth remain unclear, though a wide range of mechanisms are implicated (46). IL-1 β has been shown to increase the Wnt/ β -catenin signaling in promoting colon tumor growth (47). In another study, IL-1 β was shown to increase stemness and invasiveness in colon cancer (48). Studies have also shown that IL-6 released by colon tumor-associated fibroblasts is critical for tumor angiogenesis, and application of anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited colonic tumor cell–stroma interactions (46).

The role of IL-12 and IL-10 as tumor promoters and inhibitors is somewhat controversial. Previously, we showed that colonic tumors and serum levels of IL-12 were high in control-diet-fed *Apc*^{Min/+} mice compared with mice fed diet containing the NSAID licoferone (35). Our present findings on inhibition of IL-12 levels by aspirin and naproxen is in line with previous publications. Some studies show that IL-10 serum levels increase over time during CRC progression, suggesting a tumor-promoting role of IL-10 in patients with CRC (49-50), whereas IL-10 appears to play a protective role in animal models of CRC. IL-10 was required for regulatory Tregs to reduce tumor burden in *Apc*^{Min/+} mice (50). Moreover, recent studies show that oral administration of IL-10 microparticles decreased polyposis in the *Apc*^{Min/+} model by suppressing the development of IL-17-producing T_{regs} (50). Thus, the present findings on the induction of IL-10 by aspirin and naproxen further support the antitumor effects of IL-10. However, the exact mechanisms by which IL-10 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 three 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 showing the profound inhibitory effects of different dosing regimens of aspirin and naproxen on AOM-induced colonic invasive adenocarcinoma reflected by decreases in PGE₂, IL-1 β , and IL-6, which are implicated in tumor cell invasiveness and stemness (50).

In summary, the present results suggest that the dosing regimen of one week on/one 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 (three weeks on/three weeks off) regimens of aspirin and naproxen. Inhibition of PCNA, proinflammatory cytokines and PGE₂, and induction of p21, p53, caspase-3, and IL-10 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 high-risk colon cancer patients 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Figure 1a. Experimental design of efficacy bioassay to test different dosing regimens of aspirin and naproxen in the AOM-induced rat colon cancer model. Male F344 rats were treated with AOM (15 mg/Kg BW, *s.c.*) once weekly for two weeks. Different dosing regimens of aspirin and naproxen, as described in the methods, were administered at the ACF/early adenoma stage and at termination 48 weeks after colonic tumors were evaluated. Figure 1b. Gross necropsy of colonic tumors, illustrating examples of control diet and different dosing regimens of aspirin and naproxen. Figure 1c. Histopathological analysis of AOM-induced rat colonic tumors. Different histological subtypes of colonic tumors were described in detail in the Methods and Results sections.

Figure 2. Effect of aspirin dosing regimens on AOM-induced colon tumor incidence in F344 rats (N=Control group: 66 Rats; Treatment groups: 36 rats)

Effect of different doses and dosing regimens of aspirin (continuous, 700 and 1,400 ppm; one week on/one week off, 700 and 1,400 ppm and three weeks on/3 weeks off, 700, 1,400 and 2,800 ppm) on AOM-induced adenoma incidence (Figure 2a); non-invasive adenocarcinoma incidence (Figure 2b); and invasive carcinoma incidence (Figure 2c). Values are Mean $\pm$ SEM. Control group, N=66; treatment groups, N=36. Differences between control and treatment groups are significant per two-tailed Fisher's Exact test.

Figure 3. Effect of aspirin dosing regimens on AOM-induced colon tumor multiplicity in F344 rats (N= Control group: 66 Rats; Treatment groups: 36 rats)

Effect of different doses and dosing regimens of aspirin (continuous, 700 and 1,400 ppm; one week on/one week off, 700 and 1,400 ppm and three weeks on/three weeks off, 700, 1,400 and 2,800 ppm) on AOM-induced adenoma multiplicity (Figure 3a); non-invasive adenocarcinoma multiplicity (Figure 3b); and invasive carcinoma multiplicity (Figure 3c). Values are Mean $\pm$ SEM. Control group, $N=66$; treatment groups, $N=36$. Differences between control and treatment groups are significant per one-tailed t -test with Welch's correction.

Figure 4. Effect of naproxen dosing regimens on AOM-induced colon tumor incidence in F344 rats (N=Control group: 66 Rats; Treatment groups: 36 rats)

Effect of different doses of naproxen (200 and 400 ppm) and dosing regimens (continuous, one week on/one week off and three weeks on/three weeks off) on AOM-induced adenoma incidence (Figure 4a); non-invasive adenocarcinoma incidence (Figure 4b); and invasive carcinoma incidence (Figure 4c). Values are Mean $\pm$ SEM. Control group, $N=66$; treatment groups, $N=36$. Differences between control and treatment groups are significant per two-tailed *Fisher's Exact* test

Figure 5. Effect of naproxen dosing regimens on AOM-induced colon tumor multiplicity in F344 rats (N=Control group: 66 Rats; Treatment groups: 36 rats)

Effect of different doses of naproxen (200 and 400 ppm) and dosing regimens (continuous, one week on/one week off and three weeks on/3 weeks off) on AOM-induced adenoma multiplicity (Figure 5a); non-invasive adenocarcinoma multiplicity (Figure 5b); and invasive carcinoma multiplicity (Figure 5c). Values are Mean $\pm$ SEM. Control group, N=66; treatment groups, N=36. Differences between control and treatment groups are significant per one-tailed *t*-test with Welch's correction

Table 1.

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

	Experimental Groups	Frequency of Dose Administration	PCNA PI-Index	p21 Expression IHC-Score (1-12)	p53 Expression IHC-Score (1-12)	Caspase-3 Expression IHC-Score (1-12)	Inflammatory cytokine levels ^a (pg/mg Protein) Mean ± SEM, N=6-9				
							IL-1β	IL-6	IL-12	IL-10	PGE ₂
1	Control Diet	--	56.7±5.2	0.5±0.21	2.1±0.30	3.1±0.51	2673±95	557±36	273±18	42±4.7	2278±88
2	Aspirin 700 ppm	Continuous	38.5±3.7 p<0.006	4.7±0.52 p<0.0001	3.8±0.6 p<0.014	6.5±1.1 p<0.008	1556±58 p<0.0001	322±23 p<0.005	185±14 p<0.0009	69±5.8 p<0.0012	1477±58 p<0.0001
3	Aspirin 1,400 ppm	Continuous	27.7±3.2 p<0.0003	7.7±1.1 p<0.0001	4.5±0.9 p<0.016	8.4±1.3 p<0.002	1163±42 p<0.0001	247±17 p<0.0001	134±11 p<0.0001	84±7.1 p<0.0001	734±43 p<0.0001
4	Aspirin 700 ppm	1-week ON/ 1-week OFF	42.3±4.1 p<0.023	3.6±0.8 p<0.002	3.9±0.7 p<0.02	5.9±0.9 p<0.01	1673±67 p<0.0001	356±29 p<0.0005	202±16 p<0.007	62±5.2 p<0.0065	1672±62 p<0.0001
5	Aspirin 1,400 ppm	1-week ON/ 1-week OFF	33.7±2.8 p<0.0015	6.9±1.2 p<0.0001	5.3±1.0 p<0.007	7.7±1.1 p<0.0015	1338±48 p<0.0001	298±23 p<0.0001	168±9.8 p<0.0001	73±6.1 p<0.0005	1073±48 p<0.0001
6	Aspirin 700 ppm	3-weeks ON/ 3-weeks OFF	47.3±4.8 p=0.1	2.9±0.9 p<0.016	1.8±0.9 p=0.38	4.2±0.8 p=0.13	2132±63 p<0.0002	429±27 p<0.009	234±18 p=0.08	54±4.2 p<0.043	1748±69 p<0.0002
7	Aspirin 1,400 ppm	3-weeks ON/ 3-weeks OFF	37.7±3.5 p<0.005	3.5±0.7 p<0.0013	2.8±0.6 p=0.15	4.5±0.7 p=0.063	1858±66 p<0.0001	377±21 p<0.0007	200±12 p<0.004	60±4.7 p<0.009	1487±57 p<0.0001
8	Aspirin 2,800 ppm	3-weeks ON/ 3-weeks OFF	32.0±4.1 p<0.0016	6.2±1.3 p<0.001	4.4±0.8 p<0.011	8.6±1.6 p<0.005	1587±49 p<0.0001	311±22 p<0.0001	172±13 p<0.0001	74±6.3 p<0.0005	845±39 p<0.0001

Mean $\pm$ SEM (N=6-9)

Values are significantly different from control diet group per *t*-test with Welch's correction

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

Table 2.

	Experimental Groups	Frequency of Dose Administration	PCNA PI-Index	p21 Expression IHC-Score (1-12)	p53 Expression IHC-Score (1-12)	Caspase-3 Expression IHC-Score (1-12)	Inflammatory cytokine levels ^a (pg/mg Protein) Mean $\pm$ SEM, N=6-9				
							IL-1 β	IL-6	IL-12	IL-10	PGE ₂
1	Control Diet	--	56.7 $\pm$ 5.2	0.51 $\pm$ 0.21	2.1 $\pm$ 0.30	3.1 $\pm$ 0.51	2673 $\pm$ 95	557 $\pm$ 36	273 $\pm$ 18	42 $\pm$ 4.7	2278 $\pm$ 88
2	Naproxen 200 ppm	Continuous	32.3 $\pm$ 3.4 p<0.0008	5.3 $\pm$ 0.61 p<0.0001	4.4 $\pm$ 0.9 p<0.011	5.8 $\pm$ 1.0 p<0.011	1438 $\pm$ 47 p<0.0001	288 $\pm$ 21 p<0.0001	168 $\pm$ 13 p<0.0003	75 $\pm$ 6.7 p<0.0005	1379 $\pm$ 51 p<0.0001
3	Naproxen 400 ppm	Continuous	22.4 $\pm$ 2.3 p<0.0001	8.9 $\pm$ 1.3 p<0.0001	5.2 $\pm$ 0.83 p<0.0009	8.8 $\pm$ 1.4 p<0.0001	943 $\pm$ 38 p<0.0001	192 $\pm$ 14 p<0.0001	97 $\pm$ 8.9 p<0.0001	108 $\pm$ 8.2 p<0.0001	696 $\pm$ 38 p<0.0001
4	Naproxen 200 ppm	1-week ON/ 1-week OFF	34.7 $\pm$ 3.8 p<0.003	4.7 $\pm$ 0.81 p<0.0001	4.2 $\pm$ 0.81 p<0.009	6.1 $\pm$ 0.94 p<0.006	1574 $\pm$ 46 p<0.0001	342 $\pm$ 26 p<0.0002	187 $\pm$ 14 p<0.0015	79 $\pm$ 6.4 p<0.0001	1482 $\pm$ 62 p<0.0001
5	Naproxen 400 ppm	1-week ON/ 1-week OFF	23.1 $\pm$ 2.6 p<0.0001	8.2 $\pm$ 1.4 p<0.0001	5.1 $\pm$ 1.0 p<0.003	8.2 $\pm$ 1.12 p<0.0003	1108 $\pm$ 31 p<0.0001	223 $\pm$ 19 p<0.0001	137 $\pm$ 11 p<0.0001	93 $\pm$ 7.1 p<0.0001	945 $\pm$ 51 p<0.0001
6	Naproxen 200 ppm	3-weeks ON/ 3-weeks OFF	45.3 $\pm$ 4.7 p=0.07	2.6 $\pm$ 0.62 p<0.0017	2.9 $\pm$ 0.72 p=0.14	3.7 $\pm$ 0.68 p=0.24	2019 $\pm$ 67 p<0.0001	408 $\pm$ 33 p<0.0051	203 $\pm$ 16 p<0.007	64 $\pm$ 5.3 p<0.004	1643 $\pm$ 58 p<0.0001
7	Naproxen 400 ppm	3-weeks ON/ 3-weeks OFF	35.2 $\pm$ 3.8 p<0.0035	7.8 $\pm$ 1.2 p<0.0001	4.0 $\pm$ 0.81 p<0.015	5.5 $\pm$ 0.83 p<0.011	1647 $\pm$ 55 p<0.0001	291 $\pm$ 17 p<0.0001	172 $\pm$ 13 p<0.0004	82 $\pm$ 6.7 p<0.0001	1167 $\pm$ 43 p<0.0001

Mean $\pm$ SEM (N=6-9)

Values are significantly different from control diet group per *t*-test with Welch's correction.