Nitric oxide–releasing aspirin and indomethacin are potent inhibitors against colon cancer in azoxymethane-treated rats: effects on molecular targets

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Abstract

Nitric oxide–releasing nonsteroidal anti-inflammatory drugs (NO-NSAID) are promising chemoprevention agents; unlike conventional NSAIDs, they seem free of appreciable adverse effects, while they retain beneficial activities of their parent compounds. Their effect on colon carcinogenesis using carcinoma formation as an end point is unknown. We assessed the chemopreventive properties of NO-indomethacin (NCX 530) and NO-aspirin (NCX 4016) against azoxymethane-induced colon cancer. Seven-week-old male F344 rats were fed control diet, and 1 week later, rats received two weekly s.c. injections of azoxymethane (15 mg/kg body weight). Two weeks after azoxymethane treatment, rats (48 per group) were fed experimental diets containing NO-indomethacin (0, 40, or 80 ppm), or NO-aspirin (1,500 or 3,000 ppm), representing 40% and 80% of the maximum tolerated dose. All rats were killed 48 weeks after azoxymethane treatment and assessed for colon tumor efficacy and molecular changes in colonic tumors and normally appearing colonic mucosa of different dietary groups. Our results suggest that NO-indomethacin at 40 and 80 ppm and NO-aspirin at 3,000 ppm significantly suppressed both tumor incidence (P < 0.01) and multiplicity (P < 0.001). The degree of inhibition was more pronounced with NO-indomethacin at both dose levels (72% and 76% inhibition) than with NO-aspirin (43% and 67%). NO-indomethacin at 40 and 80 ppm and NO-aspirin at 3,000 ppm significantly inhibited the colon tumors’ (P < 0.01 to P < 0.001) total cyclooxygenase (COX), including COX-2 activity (52–75% inhibition) and formation of prostaglandin E2 (PGE2), PGF2α, and 6-keto-PGF1α, and TxB2 from arachidonic acid (53–77% inhibition). Nitric oxide synthase 2 (NOS-2) activity and β-catenin expression were suppressed in animals given NO-NSAID. In colonic crypts and tumors of animals fed these two NO-NSAIDs, there was a significant decrease in proliferating cell nuclear antigen labeling when compared with animals fed the control diet. The results of this study provide strong evidence that NO-NSAIDs possess strong inhibitory effect against colon carcinogenesis; their effect is associated with suppression of COX and NOS-2 activities and β-catenin levels in colon tumors. These results pave the way for the rational design of human clinical trials. [Mol Cancer Ther 2006;5(6):1530–8]

Introduction

Colorectal cancer, one of the most common malignancies in the Western world, accounts for nearly 60,000 deaths annually in the United States (1). Chemoprevention represents an important and feasible option for this cancer, especially in view of the limited efficacy of currently available treatments for its advanced stages. The concept that nonsteroidal anti-inflammatory drugs (NSAID) prevent colon cancer (2) was formally established through a series of recent interventional clinical trials (3) and represents a major advance in the field of cancer prevention. The widespread application of NSAIDs to cancer prevention is, however, hampered by their limited efficacy (<50%) and clinically significant side effects; the latter assume greater importance for chemoprevention, which requires their long-term administration (4).

The novel nitric oxide–donating NSAIDs (NO-NSAID) carry the promise of higher potency and greater safety compared with their conventional counterparts (reviewed in ref. 5). They consist of a conventional NSAID, which bears covalently attached a moiety that ultimately releases NO. Although the rationale for their development was that the NO that they release would compensate for the inhibition of prostaglandin synthesis by its NSAID moiety, the mechanism of their apparent gastroprotection seems more
complex. Similarly, the mechanism of their enhanced potency has not been fully clarified. Converging lines of evidence underscore the vital role of the NO-releasing moiety, and a recent report showed an excellent correlation between the amount of NO released by NO-NSAIDs and their ability to inhibit the growth of cultured colon cancer cells (6). In vitro data have shown that NO-NSAIDs have been invariably more potent than their parent compounds, although their enhanced potency ranged between 10- to >1,000-fold (7). Animal studies have shown that NO-NSAIDs suppress the formation of azoxymethane-induced colonic aberrant crypt foci (8, 9) and intestinal polyps in Min mice (10).

Although the evidence provided by the studies with Min mice or those using aberrant crypt foci as an end point is highly suggestive, the preclinical evaluation of NO-NSAIDs regarding their efficacy in colon cancer prevention requires a study using the formation of colon adenocarcinoma as an end point. To this end, we evaluated the chemopreventive efficacy of NO-aspirin and NO-indomethacin, two NO-NSAIDs (Fig. 1) whose parent compounds belong to distinct chemical NSAID classes (salicylic acid derivatives and indole acetic acids, respectively; ref. 11). Of note, in cell culture systems, NO-indomethacin was 10-fold more potent than NO-aspirin in inhibiting the growth of colon cancer cells (7). The animal model system that we used in the present studies was the Fisher 344 rats, which when treated with the carcinogen azoxymethane develop colon tumors recapitulating important features of human colon cancer (reviewed in ref. 12). This model system has been highly informative in assessing potential chemopreventive agents.

In addition to their chemoprevention efficacy, we assessed important aspects of the mechanism of action of these two compounds, including their kinetic effect on colon epithelial cells and their effect on molecular targets related to colon carcinogenesis, including cyclooxygenase (COX) isozymes, nitric oxide synthase 2 (NOS-2 or inducible isoform), and β-catenin.

Materials and Methods

Animals, Diets, Carcinogen, and NO-NSAIDs

Azoxymethane (CAS:25843-45-2) was purchased from Midwest Research Institute (Kansas, MO). Weanling male F344 rats were from Charles River Breeding Laboratories (Kingston, NY). NO-aspirin (NCX-4016) and NO-indomethacin (NCX-530) were provided by Nicox SA (Sophia-Antipolis, France) through the National Cancer Institute repository. The purity of these agents, ascertained by high-performance liquid chromatography analysis, was ≥98% (9). All ingredients of the semipurified diet (Dyets, Inc., Bethlehem, PA) were stored at 4°C before its preparation. The composition of the high-fat semipurified diet was as follows: 20% casein, 0.3% DL-methionine, 52% corn starch, 13.2% dextrose, 5.0% alphacel, 5.0% corn oil, 3.5% mineral mix, 1.0% vitamin mix, and 0.20% choline bitartrate. NO-NSAIDs were incorporated into the diet with a V-blender; their uniform distribution in the diet was monitored as described (13). Control and NO-NSAID–containing diets were prepared weekly in our laboratory and were stored in a cold room. The NO-NSAIDs were stable in the diet under our experimental conditions, as determined periodically in multiple samples by high-performance liquid chromatography; their recovery from the diet was >96%.

Determination of Maximum Tolerated Dose of NO-NSAIDs

To estimate the appropriate dose level for the efficacy study, the maximum tolerated dose (MTD) was determined in male F344 rats by feeding them various concentrations of NO-aspirin and NO-indomethacin in a 6-week toxicity study. MTD is defined as the highest dose that causes no more than a 10% body weight decrement compared with the appropriate control group and does not produce mortality or any external signs of toxicity that would be predicted to shorten the natural life span of the animal. At 7 weeks of age, groups of male F344 rats (six per group) were fed the experimental diets containing 0, 375, 750, 1,500, 3,000, or 6,000 ppm of NO-aspirin or 0, 50, 100, 200, 300, or 400 ppm NO-indomethacin. Body weights were recorded twice weekly for 6 weeks. All animals were examined daily for signs of toxicity, such as ill appearance, circling rashes, tremors, roughened coat, rhinitis, chromodacryorrhea, and prostration. At the end of 6 weeks, all animals were sacrificed, and their oral cavity, colon, small intestine, stomach, liver, and kidneys were examined for any abnormalities under a dissection microscope.

Efficacy Study

Two dose levels of each NO-NSAID were evaluated for their chemoprevention efficacy. The MTD study showed that >3,000 ppm NO-aspirin and 100 ppm NO-indomethacin do not produce any body weight loss or other evidence of toxicity in male F344 rats. Studies were designed to

![Figure 1](https://mct.aacrjournals.org/) Structures of NO-aspirin (NCX-4016) and NO-indomethacin (NCX-530).
determine the efficacy of 1,500 and 3,000 ppm NO-aspirin and 40 and 80 ppm NO-indomethacin, each given during the post-initiation stage of azoxymethane-induced colon carcinogenesis.

Male F344 rats, received at weaning, were quarantined for 10 days and had unrestricted access to modified AIN-76A control diet. Following quarantine, all rats were randomly distributed by weight into various groups (Fig. 2; Table 1) and transferred to an animal holding room. They were housed in plastic cages with filter tops (three per cage) under controlled conditions of a 12-hour light and dark cycle at 50% relative humidity and at 21°C. At 8 weeks of age, animals intended for carcinogen treatment received 2 weekly s.c. injections of azoxymethane (15 mg/kg body weight). Vehicle-treated groups (12 rats per group) received, instead of azoxymethane, an equal volume of normal saline. Two weeks after the second injection of azoxymethane or normal saline, rats were placed on control diet or diets containing NO-aspirin or NO-indomethacin as outlined in Fig. 2 and Table 1. Body weights were recorded every 2 weeks until the 16th week and then every 4 weeks until termination of the experiment, which was 52 weeks after the last azoxymethane treatment. Moribund animals were sacrificed and necropsied. All organs, including the intestine, were examined grossly under the dissection microscope. Colon tumors with a diameter of >0.4 cm were cut into halves; one half was quickly frozen in liquid nitrogen and stored at −80°C until analyzed for the expression and activity of COX isoforms, NOS-2, β-catenin, and proliferating cell nuclear antigen (PCNA), and the other was fixed in 10% neutral buffered formalin for histopathologic evaluation (14).

Analysis of NOS-2, COX Isoforms, β-Catenin, and PCNA

Most colonic tumors were adenocarcinomas and thus we used only those for the analysis of these variables. Samples were prepared as previously described (15).

Western Blot Analyses. Purified proteins and antibodies to COX-1, COX-2, and NOS-2 were from Cayman Chemicals (Ann Arbor, MI), and to β-catenin and PCNA were from Santa Cruz Biotechnology (Santa Cruz, CA). Proteins were separated on 8% PAGE-gels and then electroblotted onto polyvinylidene difluoride membranes as described (16). After blocking in 5% nonfat dry milk, these membranes were incubated overnight with primary antibodies, washed thrice, and incubated once more with the appropriate secondary horseradish peroxidase–linked antibody, developed in an enhanced chemiluminescence system and exposed to Kodak XAR5 film. Band intensities were determined using a computing densitometer.

Total COX and COX-2 Synthetic Activity. COX activities in colon tumor samples (six to eight per group) were assayed using our previously published method (13, 15). Briefly, the microsomal pellet was resuspended in 50 mmol/L potassium phosphate buffer (pH 7.4). To determine total COX activity, 150 μL reaction mixture containing 12 μmol/L [14C]arachidonic acid (420,000 dpm), 1 mmol/L epinephrine, 1 mmol/L glutathione in 50 mmol/L phosphate buffer, and 25 to 35 μg of tumor microsomal protein were incubated at 37°C for 15 minutes. To determine COX-2 activity, the reaction mixture was preincubated with 150 μmol/L aspirin to block COX-1 activity and to modify COX-2 activity. After incubation, the reaction was terminated by adding 40 μL of 0.2 mol/L HCl. The COX metabolites of arachidonic acid were extracted thrice with 0.5 mL of ethyl acetate. The combined extracts were evaporated to dryness under N2, redissolved in chloroform, and subjected to TLC on Silica G plates. The TLC plates were developed in a solvent system containing a mixture of chloroform/methanol/acetic acid/water (100:15:1.25:1, v/v/v/v) and were exposed in an iodide chamber for 5 minutes to visualize the standards. The metabolites of [14C]arachidonic acid corresponding to prostaglandin E2 (PGE2), PGF2α, PGD2, 6-keto-PGF1α, and TXB2 were detected by their comigration with authentic standards for total COX activity and [14C]-15-(R)-hydroxyeicosatetraenoic acid for COX-2 activity.

NOS-2 Activity. To quantify NOS-2 (calium independent) activity, conversion of l-arginine to l-citrulline was measured as described previously (15, 17). Briefly, the assay was carried out by adding 100 μg of sample protein to 150 μL of assay buffer [50 mmol/L HEPES, 1 mmol/L DTT, 1 mmol/L MgCl2, 5 mg/L pepstatin A, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 3 mg/L aprotinin (pH 7.4)] containing 70 μmol/L arginine, 250,000 dpm l-[3H]arginine, 2 mmol/L NADPH, 5 μmol/L tetrahydrobiopterin, 5 μmol/L flavin adenine dinucleotide, and 0.5 mmol/L CaCl2 to measure total NOS activity, or in the presence of 1 mmol/L EGTA (without calcium) to determine Ca2+-independent NOS-2 activity. After 30 minutes at 37°C, the reaction was stopped with 100 μL of 1 mol/L trichloroacetic acid. The samples were adjusted to pH 4.6 by adding 500 μL of 20 mmol/L HEPES and applied to Dowex AG 50W-X8 resin columns. l-[3H]citrulline was eluted and separated using TLC. Radioactivity was counted with a BioScan Radiomatic detector. Results were expressed as pmol l-[3H]citrulline/mg protein/min.
Table 1. Chemopreventive effect of NO-aspirin and NO-indomethacin on azoxymethane-induced colon adenocarcinoma formation in male F344 rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. rats</th>
<th>Colon tumor incidence (%)</th>
<th>Colon tumor multiplicity</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Noninvasive</td>
<td>Invasive</td>
</tr>
<tr>
<td>Carcinogen treated</td>
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<tr>
<td>Control diet</td>
<td>36</td>
<td>24/12 (66.6)</td>
<td>12/24 (33.3)</td>
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<tr>
<td>NO-aspirin (1,500 ppm)</td>
<td>36</td>
<td>20/16 (55.5)</td>
<td>3/33 (9.1)</td>
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<tr>
<td>NO-aspirin (3,000 ppm)</td>
<td>36</td>
<td>8/28 (28.6)</td>
<td>7/29 (19.4)</td>
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<tr>
<td>NO-indomethacin (40 ppm)</td>
<td>36</td>
<td>10/26 (27.7)</td>
<td>5/31 (16.1)</td>
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<tr>
<td>NO-indomethacin (80 ppm)</td>
<td>36</td>
<td>7/24 (29.2)</td>
<td>4/30 (13.3)</td>
</tr>
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<td>Vehicle treated</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control diet</td>
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<td>0</td>
</tr>
<tr>
<td>NO-aspirin (3,000 ppm)</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NO-indomethacin (80 ppm)</td>
<td>12</td>
<td>0</td>
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</tr>
</tbody>
</table>

*Both invasive plus noninvasive colon adenocarcinomas.

†% Rats with tumors (tumor incidence).

†Mean ± SE (tumor multiplicity).

Values are not significantly different from control group.

Values are significantly different from control group by χ² test.

Values are significantly different from control group by unpaired t test.

Immunohistochemistry

To evaluate the effect of NO-NSAIDs on colonocyte proliferation, we assessed PCNA expression in colonic crypt sections by immunohistochemistry, as described (16). Briefly, paraffin-embedded colons were cut longitudinally to 4-μm-thick sections and mounted on microscopic slides. After deparaffinization, sections were blocked for endogenous peroxidase activity and incubated with 1% milk. PCNA antibody (PharMingen, San Diego, CA) was applied 1:200 dilution for 1 hour at room temperature, then washed and incubated with secondary anti-rabbit IgG for 30 minutes, and then washed and incubated with avidin-biotin-complex reagent (Vector Laboratories, Burlingame, CA). After rinsing with PBS, the slides were incubated with the chromogen 3,3′-diaminobenzidine for 3 minutes, then rinsed, and counterstained with hematoxylin. Scoring was done by two investigators blinded to the identity of the samples who scored at least 30 crypts per colon (light microscopy at 400 magnification). Cells with a brown nucleus were considered proliferative positive. The proliferation index was determined by dividing the number of positive cells per crypt by the number of cells of the entire crypt or each of its compartments (upper, middle, and lower) and multiplying by 100.

Statistical Analyses

Differences in body weights among the groups were analyzed by ANOVA. Tumor incidences (number of rats with tumors) among the dietary groups were compared by the χ² method. Tumor multiplicity (total number of tumors per rat) and protein expression and activities and the proliferation index were analyzed by unpaired t test with Welch’s correction. The dose-response effect was analyzed by linear correlation (r) and regression (r²) analysis. Differences were considered statistically significant at P < 0.05.

Results

Determination of MTD and Selection of Drug Doses

NO-aspirin (6,000 ppm), given for 6 weeks under our protocol, reduced the body weight of the animals by 11% compared with controls. NO-aspirin (≤5,000 ppm) did not produce any body weight loss in the rats. NO-indomethacin (300 and 400 ppm) produced significant toxicity, and all the rats were dead by the end of the 3rd week. All the animals fed 200 ppm survived but with significantly (30%) reduced body weights. NO-indomethacin (≤100 ppm) had no significant effect on body weight, and no other toxicity was observed at the end of 6 weeks. Based on these findings, we selected 1,500 and 3,000 ppm NO-aspirin and 40 and 80 ppm NO-indomethacin for the efficacy study.

NO-Aspirin and NO-Indomethacin Lack Overt Toxicity

NO-aspirin and NO-indomethacin had no apparent adverse effects on the rats during their 52 weeks of administration at the doses used in the efficacy study, as described above. The body weights of all rats fed diets containing various levels of NO-aspirin and NO-indomethacin were comparable with those of the corresponding control groups throughout the study (data not shown). Chronic administration of NO-aspirin (3,000 or 1,500 ppm) or NO-indomethacin (80 or 40 ppm) produced no gastrointestinal erosions or other signs of toxicity nor any gross changes indicative of toxicity in several organs that we examined. This was true whether the animals were treated with azoxymethane or saline.

NO-Aspirin and NO-Indomethacin Decrease the Incidence and Multiplicity of Colon Adenocarcinomas

As expected from previous studies with this well-established tumor model, azoxymethane induced only
colon tumors, and saline-treated animals had no tumors. In azoxymethane-treated animals fed the control diet, >96% of the colon tumors were adenocarcinomas, with the rest being adenomas. The noninvasive adenocarcinomas (two thirds of the total) were growing toward the intestinal lumen and did not invade the muscularis mucosa. The invasive adenocarcinomas (one third of the total) were mostly of the signet-ring mucinous type, invading the muscularis mucosa deep into the intestinal wall and beyond. The very low numbers of adenomas precluded any further analysis of this type of neoplasms.

Table 1 summarizes our findings on colon adenocarcinomas. None of the saline-treated animals fed the control or experimental diets with NO-NSAIDs developed any colon tumors or any detectable lesions. In contrast, azoxymethane led to the formation of colon tumors, with 75% incidence and 1.58 ± 0.23 (mean ± SE for this and all subsequent values) multiplicity in animals fed the control diet. NO-aspirin reduced both the incidence and multiplicity of colon cancer in a dose-dependent manner when compared with the corresponding control values. Thus, NO-aspirin (1,500 ppm) inhibited colon adenocarcinoma incidence by 18.5% (P < 0.05, not significant) and multiplicity by 43.7% (P < 0.008), whereas the corresponding values at 3,000 ppm were 48.3% (P < 0.002) and 73.4% (P < 0.0001), respectively. NO-indomethacin displayed a similar dose-dependent reduction of both the incidence and multiplicity of colon adenocarcinoma compared with control. NO-indomethacin (40 ppm) reduced the incidence by 59% (P < 0.0002) and the multiplicity by 71.5% (P < 0.0001), whereas the corresponding values for 80 ppm were 68.7% and 76% (P < 0.0001 for both), respectively.

We analyzed the effect of these compounds on the formation of invasive and noninvasive adenocarcinomas (Table 1). NO-aspirin (1,500 ppm) failed to significantly suppress the incidence or multiplicity of noninvasive adenocarcinomas (P > 0.05 for both) but had a significant effect on the invasive ones, reducing these variables by 72.6% (P < 0.009) and 43.7% (P < 0.008), respectively. The higher dose of NO-aspirin (3,000 ppm) reduced significantly (to a greater degree than the lower dose) both the incidence and multiplicity of noninvasive adenocarcinomas (57%, P < 0.03 and 73.4%, P < 0.0001, respectively) compared with control. Paradoxically, and in contrast to the effect of the lower dose, the incidence of invasive adenocarcinomas was not significantly reduced (41.7%), whereas the reduction in multiplicity (54.5%) was significant (P < 0.04) and greater than that of the lower dose.

NO-indomethacin significantly (P < 0.0001–0.05) suppressed both the incidence and multiplicity of invasive and noninvasive adenocarcinoma compared with rats fed the control diet. This effect was only weakly dose dependent. The lower dose of NO-indomethacin (40 ppm) reduced the incidence and multiplicity of noninvasive adenocarcinoma by 58.4% and 72.45%, respectively, and the higher dose (80 ppm) by 69.2% and 77.2%, respectively. In terms of noninvasive carcinoma, the corresponding effects of the lower dose were 51.7% and 68.2% for the invasive and 68.2% and 72.7% for the noninvasive adenocarcinomas.

Formal analysis of the dose dependency of these results using linear correlation revealed that only the effect of NO-aspirin on colon tumor multiplicity was dependent on drug dose in a statistically significant manner (r = 0.994, P < 0.0001). Finally, administration of NO-aspirin and NO-indomethacin significantly reduced colon tumor volume compared with control (data not shown).

**Effect on Colonocyte Proliferation**

The effect of any chemopreventive agent on cell kinetics is an important indicator of its mode of action; in its essence, neoplasia represents an abnormal accumulation of cells. Thus, we determined by immunohistochemistry (PCNA expression) the levels of proliferation in rat colonic tumors. Figure 3 summarizes cell proliferation results as measured by PCNA-overexpressing cells. As anticipated, azoxymethane treatment increased PCNA compared with vehicle-treated animals (data not shown). Administration of NO-aspirin (1,500 ppm) inhibited the proliferation index, but this inhibition did not reach statistical significance; in contrast, the high dose of NO-aspirin significantly suppressed azoxymethane-induced colonocyte proliferation when compared with the control diet. NO-indomethacin (40 and 80 ppm) significantly suppressed PCNA expression in a dose-dependent manner.

**NO-Aspirin and NO-Indomethacin Inhibit COX-2**

Overexpression of COX-2 is an important feature of colon carcinogenesis, although its exact role in it has not yet been fully appraised (18). The animal tumor model used in this study reflects the pattern of human COX-2 expression (19, 20). To assess the potential interaction of these two NO-NSAIDs with COX isozymes, as part of their mechanism of action, we determined their effect on
both the expression and enzymatic activity of COX-1 and COX-2 in the colonic mucosa of azoxymethane/NO-NSAID–treated rats.

Our results indicate that, compared with control group, administration of NO-aspirin or NO-indomethacin did not produce any significant effect on the expression of COX-1 in colonic mucosa or in tumors. As expected (19), COX-2 was overexpressed in azoxymethane-induced colonic tumors. In animals fed high doses of NO-aspirin or NO-indomethacin, the COX-2 expression levels in colon tumors were reduced modestly when compared with the control diet group (data not shown). We have also investigated whether and to what extent NO-aspirin and NO-indomethacin modulated the formation of eicosanoid through the combined action of the COX isoforms (Fig. 4) or through COX-2 alone (Fig. 5). As summarized in Table 2, administration of NO-aspirin suppressed total COX-mediated eicosanoid metabolite formation in a dose-dependent manner (35–65%, \( P < 0.001–0.0001 \)). Administration of NO-indomethacin (40 and 80 ppm) suppressed total COX activities by >70% (\( P < 0.0001 \)). Administration of NO-aspirin and NO-indomethacin significantly inhibited COX-2 activity in a dose-dependent manner: 30% at the low dose (\( P < 0.05 \)) and 76% at the high dose (\( P < 0.0005 \)). Among COX metabolites, we observed that PGE\(_2\) and PGD\(_2\) were the major metabolites in colon tumors of rats fed the various diets.

**NO-Aspirin and NO-Indomethacin Inhibit NOS-2**

Figure 6 summarizes the effect of the two NO-NSAIDs on azoxymethane-induced colonic tumor NOS-2 activity. Both NO-aspirin and NO-indomethacin suppressed NOS-2 activity in a dose-dependent manner in these rats compared with control. For the two doses of NO-aspirin, this reduction was 38% and 47%, respectively, and for NO-indomethacin, 31.2% and 54.7%, respectively. Neither compound had any significant effect of these two NO-NSAIDs on NOS-2 protein expression levels in colonic tumors.

**Discussion**

Our results document that NO-aspirin and NO-indomethacin suppress colon adenocarcinoma formation in azoxymethane-treated F344 rats, an established model of colon cancer, extensively used in drug efficacy studies. Previous studies have suggested the potential usefulness of NO-NSAIDs in colon cancer prevention as well as the possibility that they may be devoid of significant side effects (21–24). Results from human colon cancer cell lines clearly suggested the antitumorigenic potential of NO-NSAIDs in colon cancer prevention as well as the possibility that they may be devoid of significant side effects (21–24). Results from human colon cancer cell lines clearly suggested the antitumorigenic potential of NO-NSAIDs and even documented their superior potency compared with their conventional counterparts (7, 25). Short-term experiments in animal models using preneoplastic lesions as surrogate efficacy markers showed that various NO-NSAIDs are effective colon cancer chemopreventive agents (9). Recently, studies by our laboratory have shown that NO-aspirin inhibits intestinal polyposis in APC\(^{Min}\) mice (10). However, despite extensive in vitro and in vivo work, no study to date has determined whether NO-NSAIDs suppress the formation of colon adenocarcinoma in an established model of colon cancer. For the first time, our results establish clearly that NO-aspirin and NO-indomethacin suppress dose dependently the development of colon cancer. Moreover, our findings indicate that NO-NSAIDs given in the diet suppress the formation of carcinogen-induced noninvasive and invasive adenocarcinomas.

**Figure 4.** Effect of NO-aspirin and NO-indomethacin on total COX activity, as measured by arachidonic acid (AA) metabolism. Control, tumors from rats fed AIN-76A diet without NO-NSAIDs.

**Figure 5.** Effect of NO-aspirin and NO-indomethacin on COX-2 activity, as measured by arachidonic acid metabolism leading to 15-(R)-hydroxyeicosatetraenoic acid in the presence of aspirin. Control, tumors from rats fed AIN-76A diet without NO-NSAIDs.

**Figure 6.** Effect of NO-aspirin and NO-indomethacin on COX-2 activity, as measured by arachidonic acid metabolism leading to 15-(R)-hydroxyeicosatetraenoic acid.
A particularly important finding was that chronic administration of NO-aspirin and NO-indomethacin does not produce any toxicity, such as weight loss or gastrointestinal ulcers. This information is highly significant in the context of recent clinical trials with COX-2 inhibitors (and perhaps those with conventional NSAIDs), suggesting increased cardiovascular risk associated with their long-term administration (26). The potential cardioprotective actions of NO-aspirin and other NO-NSAIDs further underscore the importance of our efficacy results. Thus, our present study lends support to the further development of NO-aspirin and NO-indomethacin for colon cancer chemoprevention.

Our MTD results indicate that when NO-aspirin and NO-indomethacin are incorporated into the AIN-76A diet their respective MTDs are >3,000 and 100 ppm. Although NO-NSAIDs have been studied extensively in cell culture systems, there are no data available for animal toxicity or MTD for these agents. Thus, our MTD studies in F344 rats provide much needed information on dose tolerability of NO-aspirin and NO-indomethacin and may assist an informed dose selection for future studies. The present MTD study did not compare NO-aspirin or NO-indomethacin to their parent compounds; however, we and others have tested a number of NSAIDs for toxicity and MTD when they were given in purified AIN-76A diet (27–29). Comparing our findings with already published data, it is apparent that NO-aspirin and NO-indomethacin are less toxic and better tolerated than conventional aspirin and indomethacin, respectively.

The rationale for the development of NO-NSAIDs has by and large been well established, and several studies have provided mechanistic evidence that supports it (25, 30–33). NO-NSAIDs seem to have a pleiotropic effect encompassing a number of effector signaling molecules that are important for colon carcinogenesis (5). Thus, we evaluated a number of such target molecules that are associated with colon tumorigenesis and/or influenced by NSAIDs.

As shown by our study, NO-aspirin and NO-indomethacin did not significantly suppress the expression of either COX-1 or COX-2 in the rats. These results are consistent with previous observations that NO-aspirin does not suppress COX expression in colon cancer cell lines (31). Our observations also suggest that dietary administration of NO-NSAIDs dose-dependently suppressed COX-mediated prostaglandin formation. In vitro studies have shown that NO-aspirin inhibits COX-1 and COX-2, and that the presence of the spacer and NO-donating moiety in the molecule slows the kinetics of COX-1 inhibition by NCX 4016 compared with aspirin (34). The positional isomer of NO-aspirin used in this study (meta) is converted essentially into salicylic acid, 3-hydroxybenzylalcohol, and a conjugated product between glutathione and the aromatic spacer linking the aspirin moiety to the NO-donating group (35, 36). In rats, following a single administration of NO-aspirin, the main metabolites in plasma were NO$_2^-$, salicylic acid, and nitrosothiols, with no intact drug observed. Of note, no direct biotransformation of

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**Table 2. Effect of NO-indomethacin and NO-aspirin on formation of total COX and COX-2 metabolites in azoxymethane-induced rat colonic tumors**

<table>
<thead>
<tr>
<th>Control diet</th>
<th>NO-aspirin (ppm)</th>
<th>NO-indomethacin (ppm)</th>
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<tr>
<td>1,500</td>
<td>3,000</td>
<td>40</td>
</tr>
<tr>
<td>Total COX</td>
<td>7,160 ± 238</td>
<td>4,850 ± 211 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>&lt; 0.0001</td>
<td>2,140 ± 135</td>
<td>1,840 ± 26 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>COX-2/15R-HETE</td>
<td>2,648 ± 108</td>
<td>2,140 ± 135 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>928 ± 74</td>
<td>628 ± 48</td>
<td>673 ± 61 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>PGF$_2$</td>
<td>1,233 ± 59</td>
<td>438 ± 33 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>366 ± 29</td>
<td>249 ± 25 (P &lt; 0.0001)</td>
<td></td>
</tr>
<tr>
<td>PGD$_2$</td>
<td>1,347 ± 63</td>
<td>444 ± 32 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>428 ± 31</td>
<td>376 ± 28 (P &lt; 0.0001)</td>
<td></td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>532 ± 51</td>
<td>356 ± 28 (P &lt; 0.013)</td>
</tr>
<tr>
<td>201 ± 21</td>
<td>166 ± 15 (P &lt; 0.0001)</td>
<td></td>
</tr>
<tr>
<td>6-Keto-PGF$_{1α}$</td>
<td>548 ± 44</td>
<td>231 ± 20 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>198 ± 17</td>
<td>153 ± 13 (P &lt; 0.0001)</td>
<td></td>
</tr>
<tr>
<td>TxB$_2$</td>
<td>578 ± 49</td>
<td>208 ± 18 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>212 ± 16</td>
<td>132 ± 10 (P &lt; 0.0001)</td>
<td></td>
</tr>
</tbody>
</table>

*Total COX-activity, pmol $^{14}$C-IAA metabolized in 15 min/mg of protein. Values are significantly lower than control diet group; by unpaired t-test.

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**Figure 6. Effect of NO-aspirin and NO-indomethacin on NOS-2 activity, as measured by arginine conversion to citrulline. Control, tumors from rats fed AIN-76A diet without NO-NSAIDs.**
NO-aspirin occurred in the upper gastrointestinal tract (37). Our study involved long-term administration of NO-aspirin, and its precise biotransformations remain unknown; there are no data available on the metabolism of NO-indomethacin. Thus, it is difficult to speculate on how NO-aspirin, far less NO-indomethacin, affected COX enzymes in our model system. Nevertheless, the significant reduction of prostaglandins and TxB2 that we observed is consistent with the notion that NSAIDs act on the catalytic activity of COX rather than its protein expression levels (38). It is important to note that COX-2-specific enzyme activity was also significantly reduced by NO-aspirin and NO-indomethacin in azoxymethane-induced colon tumors. A point that must be kept in mind in evaluating these results is that our system did not assess in situ rates of these enzymes; rather, it evaluated their catalytic potential when presented with their substrate.

Administration of these two NO-NSAIDs suppressed dose dependently the activity of NO-2, whereas they had no significant effect on its expression levels. The role of NO-2 in colon carcinogenesis is well established; supportive data include its overexpression in colon tumors (33) and inhibition of azoxymethane-induced colon carcinogenesis by NOS-2-selective inhibitors (15, 17). In vitro studies suggest that agents, such as NO-aspirin, suppress NO-2 catalytic activity and its protein expression (39). The dual inhibition in colon tumors of NO-2 and COX, two enzymes considered of great importance colon cancer chemoprevention, by these NO-NSAIDs suggests that these compounds may possess an advantage compared with specific inhibitors of each enzyme alone. This is of particular importance given the increasing evidence of interplay between NO-2 and COX-2 signals that promote colon tumorigenesis (33, 40–42). This notion is supported by our recent studies, clearly suggesting that combined application of NO-2- and COX-2-selective inhibitors provides better inhibition of azoxymethane-induced colon carcinogenesis than either agent alone (15).

Our study also indicates that NO-NSAIDs suppress the expression of β-catenin and PCNA in colon tumors when compared with rats fed the control diet. These results are consistent with our recent observations that NO-aspirin suppresses Wnt signaling in colon cancer cell lines (30). The role of β-catenin in colon carcinogenesis is well established, with dysfunction of APC or upstream Wnt-signaling molecules or mutations in β-catenin, leading to excessive colonocyte proliferation (43, 44). Thus, inhibition of β-catenin expression may contribute, at least in part, to the mechanism by which NO-NSAIDs suppress colon tumorigenesis in the rats. It is of interest that our study has shown suppression of cell proliferation, a finding consistent with the antiproliferative effect of NO-NSAIDs in various cancer cell lines (31, 45).

Of note, recent in vitro work has indicated that NO-aspirin has a major effect on the mitogen-activated protein kinase pathway affecting the activation of c-Jun and activating transcription factor-2, two proteins that participate in the formation of the activator protein complex (46).

In addition induction of oxidative stress by NO-aspirin may also be an important mechanistic effect of NO-aspirin (47). These changes may account, to a significant degree, for the cell kinetic effect of NO-aspirin. However, at this stage, it is very difficult to integrate into a comprehensive working mechanism the changes in various molecular targets that are induced by NO-aspirin, most of which, unlike the present study, have been observed in cultured cells.

The data have clear implications for human colon cancer prevention. Although preclinical data can never safely predict the response of a given agent in humans, it is fair to say that based on these data, NO-aspirin and NO-indomethacin deserve further evaluation for their eventual application to humans. Our results are consistent with in vitro and in vivo findings regarding two of NO-NSAIDs' cardinal features: enhanced efficacy against cancer and lack of appreciable toxicity. These features are critical for any agent under development for cancer chemoprevention. The two NO-NSAIDs that we studied amply meet these two criteria, as they can be assessed at this stage of their evaluation.

In summary, our results provide compelling evidence supporting the efficacy of NO-aspirin and NO-indomethacin against the development of colon adenocarcinoma in an established animal tumor model. These agents suppress both invasive and noninvasive adenocarcinomas of the colon and exhibit no gastrointestinal ulcerations or other side effects. In addition, our studies suggest that these NO-NSAIDs influence the catalytic activity of NO-2, of the two COX isoforms, and of the expression of β-catenin and PCNA in azoxymethane-induced colon tumors. These studies support the development of NO-NSAIDs for colon cancer prevention and treatment.

Acknowledgments

We thank the Technical Staff of the Research Animal Facility at the American Health Foundation Cancer Center, Dr. Malisetty V. Swamy and Cooma Indrani for their help with the animal experiments, and Julie DeVore for assistance in the preparation of this article.

References

Nitric oxide–releasing aspirin and indomethacin are potent inhibitors against colon cancer in azoxymethane-treated rats: effects on molecular targets


Mol Cancer Ther 2006;5:1530-1538.