Chemopreventive Efficacy of Combined Piroxicam and Difluoromethylornithine Treatment of Apc Mutant Min Mouse Adenomas, and Selective Toxicity against Apc Mutant Embryos

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ABSTRACT

Genetic knockout or pharmacological inhibition of cyclooxygenase-2 decreases the number and size of adenomas in mouse models of familial adenomatous polyposis. Epidemiological and clinical studies in humans indicate that the entire class of nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit both COX-1 and COX-2 enzymes are promising colon cancer chemopreventive agents. We used the Apc mutant Min mouse model to test combinations of agents that might maximize preventive benefit with minimal toxicity because they act via different mechanisms. Min mice (n = 144) were exposed to low doses of the nonselective COX inhibitor piroxicam and the ornithine decarboxylase (ODC) inhibitor difluoromethylornithine (DFMO), beginning at the time they were weaned and continuing throughout the duration of the experiment.

Piroxicam at 12, 25, and 50 ppm in the diet caused dose-dependent decreases in the number of tumors in the middle and distal portions of the small intestine. This decrease in tumor multiplicity was associated with a striking decrease in the size of those tumors that did grow out. In contrast, none of the doses of piroxicam alone decreased tumor multiplicity in the proximal portion of the intestine (duodenum). Exposure to DFMO (0.5 or 1.0% in water) caused a dose-dependent decrease in tumor multiplicity in the middle and distal portions of the small intestine. However, this decreased multiplicity was not associated with a striking decrease in the size of the tumors. Combined treatment of mice with piroxicam plus DFMO was much more effective than either agent alone and resulted in a significant number of mice totally free of any intestinal adenomas (P < 0.001), in contrast to the 100% incidence and high multiplicity in control Min mice. In addition to this profound effectiveness in reducing tumor number, the few residual tumors in mice treated with the combined drugs were markedly smaller in size than tumors that arose from control Min mice. These experiments suggest that selective COX-2 inhibition combined with ODC inhibition is a very promising approach for colon cancer prevention.

These COX-2 and ODC inhibitor drugs were not overtly toxic at the doses used when administered to mice after weaning. However, when treatment was begun in utero, the Mendelian expected progeny ratio of 1:1 that we routinely obtained in untreated control litters was no longer observed. Apc<sup>Min/+</sup> progeny of pregnant dams treated with piroxicam and/or DFMO were reduced in number and their ratio to Apc<sup>+/−</sup> progeny was decreased to <sup>0.28</sup>1. Thus, these agents are effective against adenomas that have homozygous mutation of the APC gene and also select against fetuses bearing a heterozygous mutation in the APC gene.

INTRODUCTION

NSAIDs<sup>3</sup> are perhaps the best studied class of chemopreventive agents with strong activity against colon tumors. The NSAIDs that block COX activity and inflammatory reactions have a proven efficacy in a wide variety of animal tumor models (1–3), including the Min model, in which mice bear a heterozygous mutation in the APC gene (4–6). Furthermore, there is substantial epidemiological data in humans that NSAIDs are effective in preventing colon cancer (7–9). Additionally, the NSAID sulindac is effective in regressing preexisting polyps in patients with familial adenomatous polyposis (10). All of the characterized NSAIDs have significant activity against the COX-1 and -2 enzymes, and it has often been proposed that inhibition of these activities account for much of the biological effects of the NSAIDs in the colon. However, the NSAIDs seem to have additional activities that may contribute to their chemopreventive efficacy (11–13). As mentioned above, we have shown that the NSAIDs are highly effective in the Min mouse model, and confirmatory data has been obtained by various other investigators (14–16).

Another specific chemical that demonstrates significant promise in preventing colon cancer is DFMO. DFMO is a highly specific suicide inhibitor and substrate analogue for the enzyme ODC (17, 18), which catalyzes the conversion of ornithine to putrescine. ODC is overexpressed in a wide variety of invasive and preinvasive neoplastic lesions (19). In fact, this enzyme may act as an oncogene inasmuch as transgenic mice overexpressing ODC have been shown to develop skin cancer (14). DFMO has previously been shown to be highly effective as a preventive agent against azoxymethane induced colon cancer in rats (1, 20), and is presently undergoing Phase II clinical trials in humans (21, 22).

Although highly effective, certain of the NSAIDs cause untoward effects, particularly GI problems. Hundreds of thousands of emergency hospital visits and thousands of deaths per year are associated with this class of agents (23). Regarding colon cancer prevention a number of potential solutions to GI side-effects arise: (a) the use of highly specific COX-2 inhibitors that do not exhibit the GI problems associated with COX-1 inhibitors (24–26); or (b) the potential use at lower doses of two different classes of agents—doses at which these agents do not exhibit significant adverse side effects. This latter approach of combining agents with nonoverlapping mechanisms and nonoverlapping toxicities has been routinely used in chemotherapy. However, it has also been applied in preclinical chemoprevention studies on both mammary and colon tumors (3, 25, 27, 28).

In the present study, we have examined the chemopreventive efficacy of DFMO, or piroxicam alone, or the combination of the two agents to prevent the development of adenomatous lesions in heterozygous Min mice that bear a mutation in codon 850 of the Apc gene (29). As a portion of this experiment, we administered piroxicam or DFMO in utero. Interestingly—although the drugs had embryotoxic properties against all of the genotypes—at these doses we observed preferential toxicity against those with Apc mutation.

MATERIALS AND METHODS

Animal Care and Breeding. Experimental protocols were all approved by the local Institutional Animal Care and Use Committee. Animals were housed...
in groups of one to five in microisolator cages under fluorescent lighting on a 12-h cycle and were weighed once a week. Mice were fed the defined synthetic diet AIN-93G with a defined L-amino acid mixture (Dyets, Inc., Bethlehem, PA) in powdered form via feeders that were changed with fresh diet weekly.

Pure tap water was available ad libitum for the duration of the experiment and was replaced weekly. Male C57BL/6J(Min+/+) mice were bred with C57BL/6J (+/+) females; then progeny were genotyped by PCR assay to determine whether they were heterozygous for the Min allele or were homozygous wild-type: Min+/+ male and +/+ female progeny were used to maintain the Min pedigree. Min+/+ female progeny (which have tumor incidences equal to males but breed less successfully) were randomly assigned after weaning to each of the treatment groups of the chemoprevention protocol.

Genotyping. The presence of the mutant allele was detected in DNA extracted from blood or skin using an allele-specific PCR assay as described previously (6). Briefly, an oligonucleotide primer (Ap-mutant) was designed so that the Min mutation [which converts codon 850 from a leucine (TTG) to amber (TAG)] is complementary to the 3‘ end of the primer and is, therefore, amplifiable, but the noncomplementary wild-type sequence does not amplify (6). An internal control was provided by a second primer at a location at which wild type and mutant do not differ. This optimized PCR technique correctly identified the genotype in 99.8% of assays as verified by repeat analysis performed whenever the phenotype seemed discordant with the presumed genotype (the presence of adenomas in a presumed +/+ mouse or their complete lack in a presumed Min+/+ mouse).

Drug Treatment. Piroxicam (CAS 36322-90-4) was purchased from Sigma Chemical Company (St. Louis, MO) and DFMO (CAS 70052-12-9) was a gift from Dr. Ajit Verma at the University of Wisconsin (Madison, WI). For the standard treatment groups (n = 144 total, 16 per group), piroxicam at the intended concentration was mixed in the diet beginning at approximately age 30 days; mice were killed at age 90 days after 2 months of treatment. Mice treated with DFMO were given water mixed with the intended concentration of that drug from age 30 to 90 days. For the in utero treatment groups (n = 120 total, ~30 per group), exposure to DFMO and/or piroxicam began at gestational age zero, indirectly via treatment of their mothers throughout pregnancy and nursing, and continued after weaning directly in the tested progeny as usual.

Tumor Scoring and Tissue Sampling. After the specified duration of treatment, animals were killed by CO2 inhalation. At the time of death, blood was collected in heparinized tubes, and plasma was immediately separated and frozen at −70°C for later a assay of drug levels and thromboxane B2. Colon and small intestine were removed in their entirety, opened longitudinally with fine scissors, and rinsed in saline. Mucosal samples were quickly obtained for ODC enzyme and polyamine assays; colon and segments of small intestine (each 4.0 cm in length) from the proximal (duodenum), middle (jejenum), and distal (ileum) small intestine were then spread on individually labeled strips of bibulous paper. They were fixed flat in 10% buffered formalin for 2 h, washed twice with 70% ethanol overnight, and then stained with 0.2% methylene blue in Kreb’s Ringer solution for approximately 30 min. Tumors were counted by an individual, unaware of the animal’s drug treatment status, using an Olympus SZH10 stereo dissecting microscope to record tumor number, location, and diameter to a precision of less than 0.1 mm.

Thromboxane B2 Assay. Blood samples were collected in chilled polypropylene test tubes coated with a solution of 4.5 mM EDTA and a prostaglandin synthetase inhibitor (10 μg/ml indomethacin). Thromboxane B2 in plasma was measured for each mouse using a RIA kit (New England Nuclear Research Products, Boston MA) as described previously (6). This rapid and sensitive RIA method involves separation of antibody-antigen complexes from free antigen by precipitation of antibody-bound tracer with polyethylene glycol in the presence of carrier immunoglobulin. After centrifugation, the supernatant containing the unbound antigen was decanted and was counted in a gamma counter. The results obtained for the standards were used to construct a dose-response standard curve from which the unknowns were read by interpolation.

DFMO Assay. DFMO in plasma was assayed by HPLC, using a procedure similar to that of Smithers (30). Serum was collected at the time of death from each of the animals in the DFMO-treated groups and the controls, stored frozen until the time of assay. Plasma samples (100 μl) were extracted with 4 volumes of methanol after addition of the internal standard, 4-amino-3-hydroxy butyric acid. The extracts were derivatized with o-phthalaldehyde (OPA) and chromatographic separation of the OPA-derivatized samples was achieved using a Waters Nova-Pak cartridge, gradient elution with a methanol/phosphate buffer solvent system, and fluorescent detection (335 nm excitation, 450 nm emission). Quantitation compares the peak height of the DFMO samples to that of the internal standard,4-amino-3-hydroxy butyric acid.

Piroxicam Assay. Piroxicam was assayed in plasma by HPLC using a method based on that of Macek and Vacha (31). Plasma standards or samples (100 μl of mouse plasma) were extracted with 0.3 ml of methanol after the addition of the internal standard tenoxicam. The extract was diluted 1:1 with HPLC mobile phase and separation was achieved on a 15-cm μBondapak CN column with an isotropic mobile phase of 30% methanol/70% (pH 2.2) phosphate buffer. Detection was by UV absorption at 360 nm. The amount of drug in samples was quantitated by comparison with a standard curve of known piroxicam concentrations.

ODC Assay. ODC activity in a soluble extract of colon mucosa was determined by measuring the release of CO2 from a radiolabeled ornithine substrate (22). Intestinal mucosal samples (total area, approximately 4 mm2) were immediately transferred to vials containing 0.5 ml of an ice-cold buffer [50 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, and 0.1 mM pyridoxal phosphate] and then homogenized and centrifuged. The assay mixture contained 20 mM Tris-Cl (pH 7.5), 0.32 mM pyridoxal phosphate, 4 mM DTT, 0.4 mM EDTA, 12 μM l-ornithine hydrochloride, 0.02% Brij-35, containing 0.25 μgCi d,L-[1-14C] ornithine hydrochloride and enzyme in a total volume of 0.25 ml. After incubating at 37°C for 60 min in 15-ml Corex centrifuge tubes equipped with rubber stoppers and center well assemblies, the reaction was stopped by adding 0.5 ml of 2 N citric acid. The incubation was continued for at least another hour to ensure complete absorption of 14CO2 by the ethanolamine: methoxyethanol (0.2 ml; 2:1 v/v) in the center well. Finally, the center well containing the ethanolamine: methoxyethanol was transferred to a vial containing 5 ml of toluene-based scintillation fluid and 1 ml of ethanol, and the associated radioactivity was determined in a liquid scintillation counter.

Polyamine Assays. The polyamines (spermine, spermidine and putrescine) were measured by the HPLC assay method of Kabra and Lee (32). Intestinal mucosal samples (approximately 3 mm2) were obtained from each of the three segments of small bowel and from areas of the colon not involved by tumors. These samples were immediately transferred to vials containing 0.5 ml of 0.2 N perchloric acid and stored in a freezer at −20°C until analysis. Specimens were homogenized by a polytron in 0.2 N perchloric acid and were then centrifuged. The supernatant was used for polyamine analysis, and the pellet was used for the determination of DNA content. Polyamine levels were expressed as pmol/μg DNA. After the addition of the internal standard (1.7 diaminoheptane), the polyamines in the acid extracts were derivatized with dansyl chloride, and interferences were removed from the derivatized samples using Bond-Elut C18 SPE columns. The derivatized polyamines were separated on a Waters 8 × 10 Novapak C18 cartridge using a gradient of 48–100% acetonitrile over 30 min against 10 mM sodium acetate buffer. Quantitation was accomplished with fluorescent detection (excitation 340, emission 515). Polyamines were quantitated by comparison of chromatographic peak areas of each polyamine with that of the internal standard.

Statistical Design and Analysis. The protocols used a one-way layout design for comparing the tumor growth in Min mice under various chemopreventive strategies. The randomization method that we used encouraged balanced recruitment to all of the treatment arms over time and diminishes the chance that treatment effects would be confounded with potential time effects (6, 33). This method increases the likelihood that experiments performed at different times will be comparable, making it possible to compare the magnitude of drug effects in different studies. Indeed, the tumor end points measured in the control groups in different experiments in the Jacoby lab during the past 7 years have been remarkably consistent. The tumor end points measured on all of the animals were tumor multiplicity, tumor diameter, and the intestinal location of each tumor along the longitudinal axis. All of the measurements were reported as the mean ± SE.

In addition to controlling for unmeasured factors, the initial balanced randomization method enables an exact assessment of statistical significance for data collected. Specifically, a test statistic, such as a one-way F statistic, was calculated from the observed data and also from a large collection of hypothetical data sets, each one formed by reshuffling the observed data according to the randomization scheme. Exact Ps were computed by compar-
ing the observed statistic with those simulated from this randomization distribution. Such analysis based on randomization makes accounting for multiple comparisons straightforward and minimizes the probability of calling any treatment significant when, in fact, no treatment differs from control. For comparison, we also calculated the traditional approximate normal theory $P$, accepted statistical significance only when both methods demonstrated $P < 0.05$.

RESULTS

**Piroxicam Drug Levels and Effects on Prostaglandin Synthesis.** We examined drug levels in blood plasma from the animals exposed to varying doses of piroxicam via the diet. The biological effects of these doses of piroxicam were assessed by measuring thromboxane B$_2$ levels in plasma, which reflects the COX-1 mediated synthesis of this prostanoid by platelets. Two observations warrant comment: (a) although serum levels of piroxicam increase with dose, the response is not linear over the range of doses examined (Table 1); and (b) we observed a significant dose-related inhibition of plasma thromboxane levels (Fig. 1), as expected for piroxicam because it is a nonspecific inhibitor of both forms of COX ($P = 0.051$ for this experiment overall).

**DFMO Drug Levels and Effects on Polyamine Synthesis.** In the case of DFMO, we observed a relatively linear dose response in terms of serum levels (Table 1). Combined treatment with both DFMO and piroxicam did not alter these plasma levels, which indicated there were no significant drug interaction effects (Table 1). The biological effect of DFMO in the target tissue was assessed by measuring levels of the polyamine putrescine, which is the initial product of ODC activity. Putrescine synthesis in the intestinal mucosa was decreased significantly by DFMO treatment (Fig. 2). There was no significant toxicity except for $9\%$ decrease in weight gain for the high-dose DFMO treatment group. DFMO inhibits ODC activity (data not shown) and its initial product putrescine to approximately one-half of control levels in both the colon and small intestine (Fig. 2), but the later products in the polyamine pathway (spermidine and spermine) are not decreased to as great an extent (data not shown).

**Piroxicam Reduces the Size and Number of Adenomas.** Mice were exposed to piroxicam through the diet beginning at the time that they were weaned and were then kept continually on piroxicam until death at age 90 days when blood plasma was collected for assay of platelet thromboxane B$_2$. The synthesis of this prostanoid is a measure of COX-1 activity. Data are shown as ng/ml, mean ± SE, $n = 16$ mice per group.

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| Drug levels in blood plasma and body weights of Apc/Min mice treated with DFMO mixed in the drinking water and/or piroxicam mixed in the diet$^a$ |
|-----------------|-----------------|-----------------|
| $n$             | Piroxicam in diet (ppm) | DFMO in water (%) | [Drug] in plasma |
|                 | Piroxicam (µg/ml) | DFMO (µM) | Body weight (g) | % control |
| 16              | 0               | 0         | 0 ± 0 | 0 ± 0 | 18.2 ± 1.2 | 100 |
| 15              | 12              | 0         | 0.9 ± 0.2 | 0.9 ± 0.2 | 21.1 ± 1.3 | 116$^b$ |
| 15              | 25              | 0.5       | 1.4 ± 0.1 | 1.4 ± 0.1 | 20.6 ± 1.3 | 114$^c$ |
| 15              | 50              | 1.0       | 2.5 ± 0.5 | 2.5 ± 0.5 | 17.8 ± 2.1 | 98 |
| 16              | 50              | 0.5       | 2.1 ± 0.1 | 2.1 ± 0.1 | 20.6 ± 1.1 | 114$^d$ |
| 15              | 100             | 1.0       | 3.1 ± 0.3 | 3.1 ± 0.3 | 16.4 ± 1.7 | 90$^e$ |
| 15              | 100             | 0.5       | 102 ± 16 | 102 ± 16 | 19.6 ± 2.5 | 108$^f$ |
| 15              | 100             | 1.0       | 184 ± 41 | 184 ± 41 | 17.6 ± 1.6 | 96 |

$^a$ Values are presented as mean ± SE.

$^b$ $P < 0.001$.

$^c$ $P < 0.01$.

$^d$ $P < 0.05$.

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![Fig. 1. Piroxicam effect on thromboxane B$_2$ in Apc/Min mouse plasma. Mice were treated with piroxicam mixed in the diet at dosages of 0, 25, 50, or 100 ppm beginning soon after weaning at age 30 days; treatment was continued until death at age 90 days when blood plasma was collected for assay of platelet thromboxane B$_2$. The synthesis of this prostanoid is a measure of COX-1 activity. Data are shown as ng/ml, mean ± SE, $n = 16$ mice per group.](image1)

![Fig. 2. DFMO effect on putrescine in Apc/Min mouse intestine. Mice were treated with DFMO mixed in the drinking water at dosages of 0, 0.5, or 1.0% beginning soon after weaning at age 30 days; treatment was continued until death at age 90 days. Mucosal samples from the distal small intestine were assayed for polyamines including putrescine, which is the initial product of ODC activity. Data are shown as nmol of polyamine/mg DNA, mean ± SE, $n = 16$ mice per group.](image2)
the end of the experiment. Piroxicam actually causes improved weight gain and increased survival compared with control mice, which suffer some morbidity related to their increasing tumor burden at advanced ages. Tumor multiplicity in the middle and distal portions of the small intestine decreased significantly in mice exposed to piroxicam at dosages of 12, 25, 50, and 100 ppm via the diet (Table 2). Adenomas in the distal small intestine had the most profound response to treatment (Fig. 3). In contrast, tumor multiplicity in the proximal portion of the small intestine seems to increase moderately, and the limited number of lesions that arise in the colon are minimally affected. Fig. 4 displays simultaneously the multiplicity (position of circles) and average size (diameter of circles) for all of the adenomas from each individual Min mouse in the proximal, middle, and distal portions of the small intestine. Piroxicam clearly decreases adenoma multiplicity; furthermore, the average size of the individual lesions is markedly smaller throughout the intestine. Piroxicam at all doses including the lowest dose of only 12 ppm has an effect, with the magnitude of reduction becoming quite striking when using doses ≥ 25 ppm. Interestingly, this profound effect on tumor size extends even to the proximal small intestine (Fig. 4) despite the fact that one sees an increase in the numbers of lesions in this region in mice treated with piroxicam.

Piroxicam causes regression of adenomas initially with a decrease in thickness (elevation or height above the normal mucosa) as observed under the dissecting microscope. Histological examination of

![Diagram](Image)

Table 2: Tumor multiplicity in small intestine of Apc/Min mice after treatment with DFMO and/or piroxicam

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Proximal</th>
<th>Middle</th>
<th>Distal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16</td>
<td>7.63 ± 1.23 (100)</td>
<td>23.75 ± 2.23 (100)</td>
<td>10.38 ± 1.56 (100)</td>
</tr>
<tr>
<td>Piroxicam 12 ppm</td>
<td>15</td>
<td>14.80 ± 1.64 (194)</td>
<td>16.20 ± 1.52 (68)</td>
<td>5.47 ± 0.88 (53)</td>
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<tr>
<td>Piroxicam 25 ppm</td>
<td>14</td>
<td>13.86 ± 2.26 (182)</td>
<td>10.93 ± 3.27 (46)</td>
<td>2.50 ± 0.69 (24)</td>
</tr>
<tr>
<td>Piroxicam 25 + DFMO 0.5%</td>
<td>15</td>
<td>9.67 ± 1.90 (127)</td>
<td>7.21 ± 1.93 (30)</td>
<td>0.80 ± 0.29 (8)</td>
</tr>
<tr>
<td>Piroxicam 50 ppm</td>
<td>15</td>
<td>13.80 ± 1.92 (181)</td>
<td>14.73 ± 2.68 (62)</td>
<td>2.60 ± 0.58 (25)</td>
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<tr>
<td>Piroxicam 50 + DFMO 1.0%</td>
<td>16</td>
<td>7.56 ± 1.91 (99)</td>
<td>5.00 ± 1.20 (13)</td>
<td>0.69 ± 0.29 (7)</td>
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<tr>
<td>Piroxicam 100 ppm</td>
<td>15</td>
<td>12.67 ± 3.12 (166)</td>
<td>10.40 ± 2.58 (44)</td>
<td>1.60 ± 0.58 (15)</td>
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<tr>
<td>DFMO 0.5%</td>
<td>15</td>
<td>6.40 ± 0.83 (84)</td>
<td>19.80 ± 2.43 (83)</td>
<td>3.67 ± 1.05 (35)</td>
</tr>
<tr>
<td>DFMO 1.0%</td>
<td>15</td>
<td>8.33 ± 1.03 (109)</td>
<td>13.80 ± 2.37 (58)</td>
<td>1.73 ± 0.42 (17)</td>
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</tbody>
</table>

* Values are presented as mean ± SE (% control).
* P < 0.01.
* P < 0.05.
* P < 0.001.
representative tumors randomly selected from each group confirmed that this flattened regressed appearance of the adenomas occurred only in the piroxicam-treated groups.

**DFMO Decreases Adenoma Multiplicity in Min Mice.** When DFMO was administered to Min mice beginning at the time that they were weaned, we observed a dose-dependent inhibition of tumor multiplicity, particularly in the distal (Fig. 4) and also the middle segments of the small intestine (Table 2). Interestingly, however, at DFMO doses (0.5–1.0%) that decreased tumor multiplicity to an extent similar to lower doses of piroxicam, there was no effect on tumor size (Fig. 4; Table 3).

**Combined Treatment with Piroxicam plus DFMO Decreases the Multiplicity and Size of Adenomas.** When piroxicam was administered to Min mice together with DFMO, we observed the most profound effects on adenoma formation. The two-dose combinations used resulted in significant 4-fold and 10-fold decreases in the multiplicity of adenomas in the distal or middle region of the small intestine (Table 2). Furthermore, the lesions in the distal region of the small intestine that remained after treatment were profoundly smaller than the tumors observed in control mice (Fig. 4; Table 3). Many mice in the combined-treatment groups had very few or no tumors, whereas all of the mice in the control group had more than 15 tumors (range, 15 to 83; mean, 43.1 ± 3.6).

**Drugs Inhibiting Adenomas Also Select against Embryos with Apc Mutation.** Lethality at an early stage of development occurs in ApcMin/Min embryos that completely lack normal Apc function due to homozygous mutation (34, 35). Therefore, the Min mouse line is maintained by crossing a mouse heterozygous for this mutation (ApcMin/+ ) with a C57BL/6J mouse that is wild type for the Apc gene (Apc+/+). This cross has produced more than 6000 live births, with a Mendelian expected ratio of 0.49 (412:425) from untreated dams and 0.50 (46:92) from dams treated with 1.0% DFMO and only 50% in those treated with 100 ppm piroxicam. Furthermore, the ratios of mutant: wild-type genotypes in the offspring treated with DFMO or piroxicam were significantly decreased to 0.26 (22:64) and 0.28 (18:46), respectively, in contrast to the Mendelian expected ratio of 0.49 (412:425) from control litters delivered at the same time (Table 4).

**DISCUSSION**

The Min mouse was initially developed almost a decade ago by Moser et al. (34) during random mutagenesis experiments, when a mouse line with interesting phenotypic characteristics was also noted to have anemia and multiple intestinal (Min) tumors. Subsequently, it was shown that these were multiple adenomas due to a germ line mutation in the Apc gene, which is itself associated with the majority of sporadic human colon cancers and is the primary gene involved in the hereditary syndrome familial adenomatous polyposis (29). This useful adenoma model has been used by a wide variety of investigators to test for potential chemopreventive agents. Perhaps the most effective class of compounds in this particular mouse model has been the NSAIDS, including piroxicam, sulindac, and aspirin (4–6, 28). It has been known for some time that the NSAIDS inhibit both the COX-1 and COX-2 enzymes, which convert arachidonic acid to prostaglandins. Because they inhibit prostaglandin synthesis, it was long ago proposed that altered prostanoid levels were the primary mechanism by which the NSAIDS exert their effects (36). Alternatives to the routine use of standard NSAIDS have been under continual investigation because all of the members of this class of drugs have a risk of toxicity and significant side effects such as GI ulceration and bleeding.

The recent development of COX-2 specific agents is a promising alternative that may not have the negative side effects primarily associated with the additional inhibition of COX-1 activity by non-selective agents such as NSAIDS (24). At least two COX-2 selective agents have proven to be relatively effective in the colon tumor models (3, 25, 28). Another alternative is to use a lower dose of an

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**Table 3** Tumor diameter in small intestine of Apc/Min mice after treatment with DFMO and/or piroxicam

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Tumor diameter in segments of small intestine</th>
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<tr>
<td></td>
<td></td>
<td>Proximal</td>
<td>Middle</td>
<td>Distal</td>
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</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>2.56 ± 0.25 (100)</td>
<td>2.24 ± 0.20 (100)</td>
<td>1.86 ± 0.14 (100)</td>
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<tr>
<td>Piroxicam 12 ppm</td>
<td>15</td>
<td>1.81 ± 0.13 (71)</td>
<td>1.45 ± 0.11 (65)</td>
<td>1.21 ± 0.07 (65)</td>
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<td>Piroxicam 25 ppm</td>
<td>14</td>
<td>1.53 ± 0.08 (60)</td>
<td>1.18 ± 0.12 (53)</td>
<td>0.94 ± 0.09 (51)</td>
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<tr>
<td>Piroxicam 25 ppm + DFMO 0.5%</td>
<td>14</td>
<td>1.36 ± 0.13 (53)</td>
<td>1.15 ± 0.14 (51)</td>
<td>0.90 ± 0.16 (48)</td>
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<tr>
<td>Piroxicam 50 ppm</td>
<td>15</td>
<td>1.38 ± 0.13 (54)</td>
<td>1.17 ± 0.11 (52)</td>
<td>0.94 ± 0.09 (51)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piroxicam 50 ppm + DFMO 1.0%</td>
<td>16</td>
<td>1.11 ± 0.10 (43)</td>
<td>0.81 ± 0.10 (36)</td>
<td>1.21 ± 0.22 (65)</td>
<td></td>
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<tr>
<td>Piroxicam 100 ppm</td>
<td>14</td>
<td>1.28 ± 0.10 (50)</td>
<td>0.93 ± 0.06 (41)</td>
<td>0.95 ± 0.16 (51)</td>
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<tr>
<td>DFMO 0.5%</td>
<td>15</td>
<td>2.97 ± 0.44 (116)</td>
<td>2.34 ± 0.12 (104)</td>
<td>1.86 ± 0.21 (100)</td>
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</tr>
<tr>
<td>DFMO 1.0%</td>
<td>15</td>
<td>2.46 ± 0.22 (96)</td>
<td>1.91 ± 0.17 (85)</td>
<td>1.66 ± 0.31 (89)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are presented as mean diameter in mm ± SE (% control).

# P < 0.01.

* # P < 0.001.

# P < 0.05.

---

**Table 4** Chemopreventive drugs embryotoxicity selects against Min mutant mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mothers treated</th>
<th>Pups born per litter</th>
<th>Apc genotypes of progeny surviving to age 20 days</th>
<th>Total progeny</th>
<th>% Min*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33</td>
<td>6.2</td>
<td>425</td>
<td>837</td>
<td>49%</td>
<td>—</td>
</tr>
<tr>
<td>Piroxicam 100 ppm</td>
<td>29</td>
<td>4.6</td>
<td>46</td>
<td>64</td>
<td>28%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DFMO 1%</td>
<td>29</td>
<td>4.5</td>
<td>64</td>
<td>22</td>
<td>26%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Piroxicam 100 ppm + DFMO 1%</td>
<td>28</td>
<td>4</td>
<td>12</td>
<td>16</td>
<td>25%</td>
<td>ns</td>
</tr>
</tbody>
</table>

* Mendelian expected = 50%.
NSAID, such as piroxicam, in combination with some other effective agent with different side effects. The agent we used here was the highly selective ODC inhibitor DFMO (18). The enzyme ODC catalyzes the conversion of ornithine to putrescine, the initial rate-limiting step in the synthesis of polyamines. ODC expression is increased in a wide variety of human cancers and preinvasive lesions and acts as an oncogene in experiments in which it is ectopically overexpressed (14). Prior preventive studies in the azoxymethane carcinogen-induced rat colon cancer model had shown that DFMO is highly effective (20, 37). DFMO has also been shown to be effective in various skin tumor models (18, 38). We have demonstrated that DFMO was moderately effective against adenoma formation in the Min mouse model. Given previous data indicating that DFMO may inhibit cellular proliferation, we expected that adenomas developing in the presence of DFMO would be much smaller than adenomas from control mice. However, there was no significant difference in tumor size after DFMO treatment. Perhaps it should not be surprising that effects on tumor size could be separate from multiplicity, because some agents may affect the growth rate independently of the initiation or establishment of adenomas. The lack of observed effect on tumor size after treatment with the polyamine pathway inhibitor DFMO contrasts sharply with the effects of piroxicam, a prostaglandin pathway inhibitor. Results with piroxicam alone were consistent with those of our previous study with that drug as a single agent, which indicated a decrease in both tumor multiplicity and size (6). The few residual adenomas remaining after treatment with piroxicam are much flatter (decreased height above the mucosal plane) and about one-half the diameter compared with adenomas from untreated control mice. Piroxicam seems to cause adenoma regression similar to effects, caused by the specific COX-2 inhibitor celecoxib, that we previously reported.

The combination of relatively low doses of piroxicam together with DFMO profoundly decreased intestinal adenomas and was more effective than either drug alone. The appropriate intermediate markers (ODC enzyme activity and polyamine levels in the intestinal tissue or thromboxane B2 in blood) were decreased by DFMO and piroxicam in parallel to their tumor inhibitory effects. Tumor multiplicity and diameter were decreased significantly, with additive effects of this drug combination on both measures of the tumor phenotype. Despite the large number of tumors in every control animal, combination therapy almost completely suppressed adenomas in many of the treated mice. Although tremendous clinical efficacy would be necessary to prevent all adenomas in patients with severe polyposis phenotypes, combination treatments such as these used in conjunction with colonoscopic polypectomy might delay or obviate the need for colectomy in some patients. After patients have total colectomy, the next concern is periampullary tumors in the duodenum. In patients with subtotal colectomy, the rectum remains at high risk, and ileal pouch procedures may have some risk of adenomas. Our studies reveal that efficacy varies greatly depending on the region of intestine: proximal-mid-distal (duodenum-jejunum-ileum). Piroxicam is much more effective in the distal small intestine (ileum). The cause for this gradient of response is unknown but could be due either to luminal substances such as biliary secretions or growth factors or to intrinsic differences in gene expression in each intestinal region. Because duodenal tumors are clinically a very important problem in humans with familial adenomatous polyposis, our results with the Min mouse model suggest that careful studies of efficacy in the proximal small intestine are needed to assess the usefulness of NSAIDs in the familial adenomatous polyposis patient population.

At the very beginning of these experiments, the authors thought that it might be necessary to treat mice starting at a very early age, when adenomas begin developing, to achieve striking efficacy. The known major genetic change associated with adenoma formation—loss of the chromosome that bears the normal APC allele—may begin occurring while the animals are in utero. We, therefore, examined the effects of both DFMO and piroxicam in this model when initially administered to the dam. Routinely, after looking at thousands of offspring, one observes a genetic ratio of 1:1 (heterozygous APC mutant mice: homozygous APC wild-type mice) in crosses between heterozygous APC mutant mice and homozygous APC wild-type mice. In contrast, in similar crosses in which the dams were treated with piroxicam or DFMO, the resulting ratios were approximately 0.3:1 (heterozygous APC mutant mice:homozygous APC wild-type mice).

This ratio, together with the decreased litter size, implies that more than 60% of mice with an APC\(^{Min/+}\) genotype were lost during development and that both of these agents, when administered in utero, were preferentially embryotoxic to mice with the mutant APC allele. Because the APC gene has not been previously shown to be directly associated with response to any chemical agents during development, the cause of this striking effect is still unknown.

In summary, these experiments demonstrate for the first time in a mouse model of adenomatous polyposis the profound efficacy of a combination-treatment approach, using relatively low doses of drugs that inhibit ODC and COX-2. Combined treatment of mice with piroxicam plus DFMO was much more effective than either agent alone and resulted in a significant number of mice totally free of any intestinal adenomas, in contrast to the 100% incidence and high multiplicity in control Min mice. In addition to this profound effectiveness in reducing tumor number, the few residual tumors in mice treated with the combined drugs were markedly smaller in size than tumors that arose from control Min mice. These experiments suggest that selective COX-2 inhibition combined with ODC inhibition is a very promising approach for colon cancer prevention.

ACKNOWLEDGMENTS

We appreciate the expert technical assistance of Marcia Pomplun.

REFERENCES


Chemopreventive Efficacy of Combined Piroxicam and Difluoromethylornithine Treatment of Apc Mutant Min Mouse Adenomas, and Selective Toxicity against Apc Mutant Embryos


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