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Advances in Brief

Chemoprevention of Spontaneous Intestinal Adenomas in the *Apc* ^{Min} Mouse Model by the Nonsteroidal Anti-inflammatory Drug Piroxicam[1]

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Abstract

C57BL/6J-*Min*/+ mice (n = 56), heterozygous for a nonsense mutation in the *Apc* gene, were randomized at weaning to seven groups, including groups treated with piroxicam at 0, 50, 100, and 200 ppm in the AIN93G diet. After only 6 weeks of treatment, intestinal adenomas and aberrant crypt foci were counted, and serum levels of piroxicam and thromboxane B_2 were quantitated. Tumor multiplicity was decreased in a dose-dependent manner from 17.3 ± 2.7 in the control to 2.1 ± 1.1 (12%) in the high-dose piroxicam group ($P < 0.001$). Thromboxane B_2 levels in plasma also decreased monotonically in parallel to the decrease in tumor multiplicity, consistent with the prostaglandin inhibitory effect of piroxicam. The Min mouse model demonstrates that the nonsteroidal anti-inflammatory drug piroxicam has strong biological and therapeutic effects, potentially useful for prevention of the early adenoma stage of tumor development.

Introduction

Adenomatous polyps are useful targets for colon cancer chemoprevention trials because they are the primary precursor lesion for the development of most colon carcinomas in humans (1). However, tumor development in the standard chemically induced animal models of colon cancer differs in several important respects from that observed in humans: (*a*) these induced carcinomas often develop from flat foci of dysplasia rather than adenomatous polyps (2); (*b*) the relatively high dosage genotoxic chemical carcinogenesis regimens probably differ from the natural etiological causes involved in most sporadic cases in humans; (*c*) the carcinogen-induced models are unlikely to reflect accurately the pathogenesis of colonic neoplasms in specific syndromes of genetic predisposition such as FAP[3] or hereditary nonpolyposis colon cancer, where mutations in *APC* or various DNA repair genes occur in the germline. Although only a relatively small percentage of human colon cancers arise in FAP kindreds, the *APC* gene that is mutated in these kindreds is also mutated early in the development of sporadic colon cancer and hereditary nonpolyposis colon cancer (3, 4). Because each of these forms of colon cancer share mutations in *APC*, an animal model with an alteration in this gene would be most appropriate for testing chemopreventive agents. We have developed a model of spontaneous intestinal neoplasia, the Min mouse, which we believe is more representative of natural adenoma development. The *Min* mutation was discovered at the University of Wisconsin by phenotypic screening after random germline mutagenesis with ethylnitrosourea (5). The *Min* mutation is an autosomal dominant heterozygous nonsense mutation of the mouse *Apc* gene, converting codon 850 from a leucine (TTG) to amber (TAG; Ref. 6). It is homologous to the *APC* mutations carried in the germline of humans with familial adenomatous polyposis or that occur somatically in the majority of sporadic colon neoplasms. The Min model is advantageous for testing chemopreventive agents targeted against early stage lesions, because scores of adenomas grow to a grossly detectable size in only 1 to 3 months on a defined genetic background (the inbred mouse strain C57BL6/J-*Min*/+; Ref. 5). Previous experiments provided evidence that NSAIDs may prevent colon cancer and/or adenomatous polyps (8–11). Although these drugs inhibit cyclooxygenase activity and decrease prostaglandin levels (9), recent evidence suggests that another mechanism, perhaps induction of apoptosis, could be involved in tumor inhibition (12). To further investigate the chemopreventive effect of NSAIDs and other drugs, we have begun a series of experiments using the Apc^{Min} mouse model. We demonstrate here that piroxicam in the dosage range of 50 to 200 ppm in the diet significantly inhibits prostaglandin levels (serum thromboxane B_2) and decreases the number of intestinal adenomas in Min mice, reducing both in parallel to less than 20% compared to controls.

Materials and Methods

Min Mouse Breeding. Male C57BL/6J-*Min*/+ mice, obtained from the original colony at the McArdle Laboratory (5), were bred with C57BL/6J (+/+) females purchased from The Jackson Laboratory. Progeny were genotyped as described below to determine if they were heterozygous for the *Min* allele or were homozygous wild type. *Min*/+ male and +/+ female progeny were used to maintain the Min pedigree, since they were more fecund for breeding purposes. *Min*/+ female progeny (which have tumor incidences equal to males; Ref. 5) were randomly assigned at weaning to each of the treatment groups of the chemoprevention protocol.

Genotyping. The presence of the mutant allele was detected by an allele-specific PCR assay for the known Apc^{Min} nonsense mutation (7). An oligonucleotide primer (*Apc*-mutant) was designed so that the *Min* mutation (underlined) is complementary to the 3′-end of the primer and is, therefore, amplifiable, but the noncomplementary wild-type sequence does not amplify. The *Apc*-mutant primer was similar to that used previously (7) but was three nucleotides longer to improve amplification efficiency. An internal control was provided by a second primer at a location where wild-type and mutant do not differ. The *Apc*-mutant and *Apc*-15 primers generated a product of 313 bp from mutant DNA. The *Apc*-9 and *Apc*-15 primers generated a product of 619 bp from either mutant or normal DNA. The primer names, oligonucleotide sequence, and location in *Apc* were: *Apc*-9, 5′-GCC ATC CCT TCA CGT TAG-3′, 2241–2258; *Apc*-mutant, 5′-TTC TGA GAA AGA CAG AAG TTA-

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[3] The abbreviations used are: FAP, familial adenomatous polyposis; APC, adenomatous polyposis coli; ACF, aberrant crypt foci; NSAID, nonsteroidal anti-inflammatory drug.

3′, 2547–2567; and *Apc*-15, 5′-TTC CAC TTT GGC ATA AGG C-3′, 2859–2841.

Drug Treatment. After weaning, animals were housed in groups of 1–5 in microisolator cages under fluorescent lighting on a 12-h cycle and were weighed once per week. Pure tap water was available *ad libitum* for the duration of the experiment and was replaced weekly. The mice were treated with drug mixed in the AIN-93G diet (Dyets, Inc., Bethlehem, PA), beginning at approximately age 30 days, and then were sacrificed after 6 weeks of treatment (ages 69–73 days).

The defined synthetic diet AIN-93G was developed as a standard diet by the *ad hoc* Committee on the Reformulation of the AIN-76A Rodent Diet, sponsored by the American Institute of Nutrition. This diet was sterilized by 0.75 MRad (7.5 kGy) gamma irradiation (Isomedix, Whippany, NJ) to eradicate microorganisms. Different lots of diet were ordered during the course of these experiments, but any batch effects were minimized by the allocation of approximately equal numbers of animals between control and treatment groups during any time interval. The chemopreventive agent piroxicam is thought to be stable for at least 7 days in a standard rodent diet at the concentrations to be used in these studies (8). We prepared new batches weekly by thorough mixing of the diet with the indicated doses of piroxicam and stored them until use in sealed containers at 4°C. Fresh diet was added to protected feeders three times weekly and was completely changed after emptying the feeders once weekly.

Tissue Sampling and Tumor Scoring. Animals were sacrificed by CO_2 inhalation euthanasia. Blood was collected at the time of sacrifice in heparinized tubes, and plasma was immediately separated and frozen at −70°C for later assay of piroxicam and thromboxane B_2. The entire colon and sample segments of small intestine (each 4.0 cm in length) were quickly removed from the proximal (duodenum), middle (jejunum), and distal small bowel (ileum). The intestinal segments were opened longitudinally with fine scissors, rinsed in saline, and then spread on individually labeled strips of bibulous paper. Tissues were fixed in 10% buffered formalin 2 h and then washed twice with 70% ethanol. Intestinal segments were examined by an individual unaware of the animal's drug treatment status, using an Olympus SZH10 stereo dissecting microscope to record tumor number and location (5). Aberrant crypts found throughout the infestional tract were quantified in each colon using a methylene blue staining technique (15). Hematoxylin and eosin-stained sections of formalin-fixed, paraffin-embedded tumors were examined microscopically for histological assessment.

Piroxicam Assay. Piroxicam in plasma was assayed by high-performance liquid chromatography using a method based on that of Macek and Vacha (16). Plasma standards or samples (50–100 μl of mouse plasma) were diluted with 1.5 volumes of methanol after the addition of the internal standard, tenoxicam. The extract was diluted with an equal volume of the mobile phase, and a 50-μl aliquot was taken for assay. Separation was achieved on a 15-cm μBondpak CN column with an isocratic mobile phase of 30% methanol/70% phosphate buffer (pH 2.2), which was pumped at 1.8 ml/min. Detection was by UV absorption at 360 nm. The plasma standard curve was linear from 0.1–5.0 μg/ml, and the variability in replicate assays was less than 5% at all concentrations.

Thromboxane B_2 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 B_2 in plasma was measured for each mouse using a RIA kit (New England Nuclear Research Products, Boston, MA). 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.

Statistical Analysis. At weaning, each of the female *Min*/+ mice was allocated to one of four different treatment arms in equal numbers ($n = 8$ for each group). The experimental groups were control and piroxicam at 50, 100, or 200 ppm in diet. The randomization schedule was prepared in advance, and unlike a standard randomization, it encouraged a balance in the allocation over time to account for potential time effects. The standard randomization scheme is equivalent to placing 32 tickets in an urn, 8 numbered by each of 4 different numbers corresponding to the 4 experimental groups. An allocation is produced by sampling without replacement from the urn. When mice are entered sequentially, there is the potential for time effects on the outcome. Rather than block on time, a more flexible solution is to have the randomization scheme encourage the filling up of treatment arms uniformly over time. We allocated mice by a scheme equivalent to placing 64 tickets in an urn, 16 numbered by each of 4 different numbers corresponding to the 4 treatment arms. Again, tickets are drawn out sequentially, without replacement, but a mouse must accumulate 2 tickets of the same number before it can be allocated to that arm. Upon allocation, it returns to the next mouse any other tickets it did not use to enter the treatment arm. The tendency, therefore, is to balance the allocation among groups over time.

To assess the significance of observed differences in tumor counts among the groups, two methods were used. A one-way F-statistic was computed and compared to its randomization distribution. To do so, a computer was instructed to produce a series of 50,000 hypothetical randomizations by the same procedure as used for the real experiment. Thus, for any particular mouse, the observed tumor counts or other measured data would remain associated with that mouse's individual identification number, but the assignment to a treatment group would be varied randomly. Under the null hypothesis of no treatment difference, each mouse's tumor count does not depend on the treatment it received, and thus a hypothetical F-statistic may be recomputed for each such randomization. Such F tests were performed on total tumor counts, separately on tumor counts in each intestinal location, and jointly to assess interaction between treatment and intestinal location.

To validate the novel statistical methods used in this study, normal theory *P* values were also computed according to standard formulas, but these computations lead to the same conclusions. All measurements are reported as the mean ± SE.

Results

There was a dramatic reduction in the number of tumors in *Min*/+ mice treated with piroxicam at all doses tested (Fig. 1; Table 1). Differences in total tumor counts among the control and treatment groups were statistically significant ($P < 0.001$). This conclusion holds separately for the three regions of the small intestine ($P < 0.001$ each) but not for the large intestine ($P = 0.6$). The pattern of these

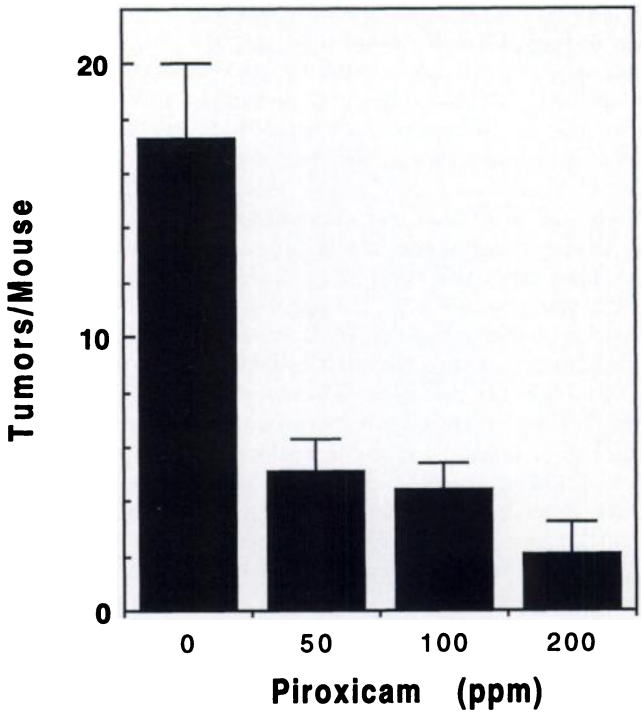

Fig. 1. Effect of piroxicam treatment (drug was added to the diet from ages 30–70 days) on the number of intestinal tumors in *Min* mice ($n = 8$ mice for each group). The total tumor multiplicity in all segments is shown, with data expressed as means (*bars*, SE). $P < 0.001$ overall and for each treatment group *versus* control.

Table 1 *Tumors in each intestinal segment in Min mice treated with piroxicam*

C57BL/6J-*Min*/+ mice (n = 56) with a heterozygous mutation in the *Apc* gene were randomized at weaning to seven groups, including the four groups shown below treated with piroxicam at 0, 50, 100, and 200 ppm in the AIN93G diet. After 6 weeks of treatment, intestinal tumors (primarily adenomas) were counted in the entire colon and sample segments of small intestine (each 4.0 cm in length). Data are presented as mean ± SE.

Intestinal location	Control diet	Piroxicam treatment[a]		
		50 ppm	100 ppm	200 ppm
Proximal[b]	2.7 ± 0.3	1.2 ± 0.3	1.1 ± 0.4	0.6 ± 0.4
Middle[b]	8.0 ± 2.1	1.6 ± 0.7	1.2 ± 0.4	0.1 ± 0.1
Distal[b]	6.0 ± 0.9	1.6 ± 0.6	0.9 ± 0.4	0.7 ± 0.7
Colon	0.6 ± 0.3	0.8 ± 0.3	1.2 ± 0.5	0.7 ± 0.3
Total[b]	17.3 ± 2.7	5.2 ± 1.2	4.5 ± 1.0	2.1 ± 1.1

[a] An F test for interaction between location and treatment indicates that the drug piroxicam does have regional differences in chemopreventive effectiveness ($P < 0.001$).
[b] Differences in total tumor counts among the control and treatment groups are statistically significant ($P < 0.001$). This conclusion holds separately for the three small intestinal locations ($P < 0.001$ each) but not for the large intestine ($P = 0.6$).

differences does not appear to be the same in each intestinal segment, as determined by an F test for interaction between location and treatment ($P < 0.001$). The drug piroxicam reduces adenoma multiplicity significantly only in the small intestine (Table 1). The total number of intestinal tumors was 5.2 ± 1.2 at 50 ppm, 4.5 ± 1.0 tumors at 100 ppm, and 2.1 ± 1.1 tumors at 200 ppm piroxicam; all were significantly different ($P < 0.001$) from the control group with 17.3 ± 2.7 tumors. At the highest dose tested (200 ppm piroxicam), tumor multiplicity was reduced to only 12% of the control group. Tumor data were unavailable for one of the animals randomized to the 200 ppm piroxicam group, since that mouse died. All presumed *Min*/+ animals with zero tumors were genotyped again to verify that they carried *Min* and were not +/+. Only one mouse (in the control group) had been assigned to the wrong genotype at the time of randomization; it was excluded from analysis.

As expected, prostaglandin levels were reduced by piroxicam treatment (Fig. 2) and decreased monotonically with dose, as did tumor multiplicity. Serum thromboxane B_2 was 3.3 ± 1.2 ng/ml in the control group, 1.3 ± 0.3 ng/ml at 50 ppm, 0.9 ± 0.3 ng/ml at 100 ppm, and 0.7 ± 0.1 ng/ml for the 200-ppm piroxicam group. These groups are significantly different ($P < 0.001$). Pairwise comparison shows that the difference is attributed to differences between the control group and each of the two highest doses of piroxicam ($P < 0.05$ each).

Piroxicam steady-state plasma concentration appears to be proportional to the dosage of drug administered in the diet up to at least 100 ppm. Piroxicam levels were 2.0 ± 1.2 μg/ml at 50 ppm, 4.1 ± 3.1 μg/ml at 100 ppm, and 2.9 ± 2.4 μg/ml in the 200-ppm group. These levels are similar to those observed in our human chemoprevention trials (data not shown). Toxicity was minimal overall, and no morbidity or mortality was observed in any mice treated with 50 or 100 ppm piroxicam. There was possible toxicity at the highest dose, since one death occurred in a mouse treated with 200 ppm piroxicam, but necropsy did not reveal a specific cause attributable to the drug. The piroxicam-treated groups gained weight at a rate equal to the control group (data not shown), and there was no apparent morbidity.

In addition to determining tumor numbers, each colon was carefully examined by methylene blue staining for ACF. The mean number of ACF per mouse in the control group was 0.14 ± 0.14, not significantly different from the treatment groups with 0, 0.25 ± 0.16, and 0.29 ± 0.18. Microscopic histopathological examination of hematoxylin and eosin-stained sections from a random sample of 20 tumors (5 from each of the 4 experimental groups) showed that 19 (95%) were adenomas, and 1 (5%) was an adenoma with severe dysplasia or carcinoma *in situ*.

Discussion

These results demonstrate the efficacy of a chemopreventive agent against adenomas developing naturally in an animal model of familial polyposis. The *Min* germline mutation of the *Apc* gene in our model is similar to the mutations observed in both sporadic and familial adenomas and carcinomas in humans. In the present experiments, tumors were counted at only one point in time, so we cannot discriminate between effects on tumor establishment and maintenance or growth and progression. However, the order-of-magnitude decrease in adenoma multiplicity indicates that there must be at least one strong effect of piroxicam on the system that is biologically and therapeutically important.

Our data demonstrate that the NSAID piroxicam, at dosages that inhibit blood plasma levels of the prostaglandin thromboxane B_2, dramatically reduces the number of adenomas in *Min*/+ mice. The magnitude of inhibition was significant, even at a low dosage of piroxicam, with a dose-response curve for tumors parallel to that observed for thromboxane B_2. Although consistent with the hypothesis that prostaglandin inhibition is the mechanism of action of NSAIDs in chemoprevention, this parallel change does not prove a cause-effect relationship. Further studies will be necessary to determine whether NSAIDs prevent adenomas primarily through cyclooxygenase inhibition. The substrate of cyclooxygenase, arachidonic acid, is produced by enzymatic conversion of membrane phospholipid by phospholipase A_2 (9). In this regard, it is of interest that a secreted form of phospholipase A_2 has recently been proposed as a candidate for a major genetic modifier of *Min*, *Mom 1*, because the *Pla2s* gene is mutated in sensitive strains and cosegregates among inbred mouse strains with the *Mom 1* locus (7). Although it would be attractive to hypothesize that changes in the synthesis of prostaglandins or digestion of their fatty acid precursors explain both the therapeutic and genetic observations, the situation is somewhat complex. B6-*Min*/+ mice carry a mutant allele at Pla2s and develop

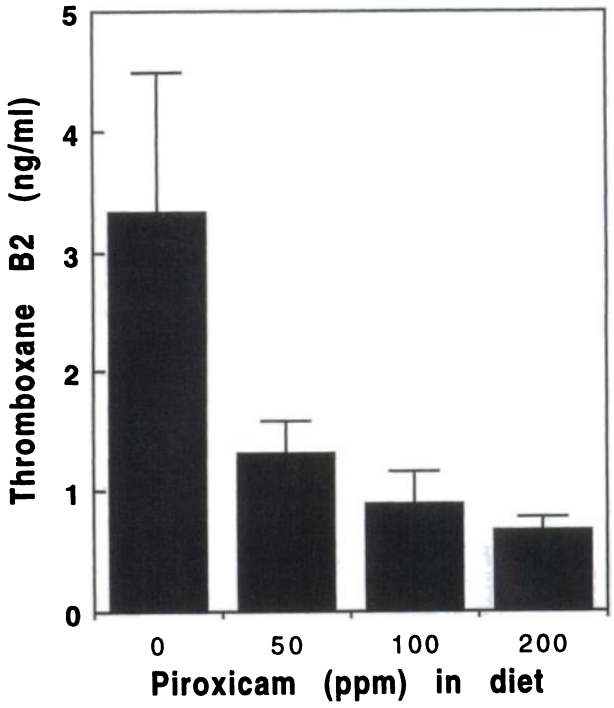

Fig. 2. Effect of piroxicam treatment (with drug added to diet from ages 30–70 days) on levels of thromboxane B_2 in blood plasma from *Min* mice (n = 8 mice for each group). Thromboxane B_2 levels (ng/ml) are expressed as means (*bars*, SE). $P < 0.001$ overall and $P < 0.05$ for the two higher dose treatment groups *versus* control.

a greater number of tumors than AKR-*Min*/+ mice and mice from several strains that carry a functional allele at Pla2s. Therefore, a simple direct effect of this enzyme on intestinal epithelial prostaglandins is unlikely to explain the differences in tumor multiplicity. Whatever *Mom 1* is, however, it is a locus that maintains heterozygosity in tumors, perhaps acting nonautonomously (18).

Our data indicate that there are regional differences in the chemopreventive effectiveness of the drug piroxicam in Min mice. There also are interesting species differences in the proximal to distal location or distribution of tumors, with Min mouse adenomas more uniformly distributed throughout the small intestine, while human adenomas primarily occur in the colon and periampullary region. Future experiments should explore the biological differences between the mouse and human, possibly contributing to these segmental differences. These include diet, bacterial flora, biliary secretions, and genetic background (including modifier genes unlinked to APC).

It is important to note that the current experiment resulted in fewer total tumors in the untreated controls (17.3 ± 2.7) than previously reported by us (29 ± 1.0; Ref. 5). Factors possibly responsible for this difference include age at sacrifice (70 *versus* 100 days), and diet (defined synthetic *versus* chow). However, the two experiments were performed in different facilities and were not part of a single, randomized trial.

Induction of apoptosis has been suggested as a mechanism for NSAID chemopreventive action, possibly independent of effects on prostaglandins (12, 17). Although the human intestinal epithelium normally is quite active with respect to both mitosis and apoptosis, in familial polyposis the crypt proliferative zone shifts upwards, and apoptosis is decreased (14). The NSAID sulindac and its metabolites appear to increase apoptosis in human HT-29 colon carcinoma cells in culture and in the rectal mucosa of humans with FAP (12). The regulation of apoptosis in the small intestine obviously could impact on the development of adenomas located there. In this regard, the negative regulator of apoptosis, *Bcl-2*, is expressed in the mouse colon but not the small intestine (13). Mice with a homozygous knockout of *Bcl-2* demonstrate enhanced apoptosis in the colon but not the small intestine (13). Apoptosis appears to be more active in the small intestine in humans, which could explain the relative paucity of tumors compared to the mouse.[4] There is a need to better understand the underlying mechanisms to improve the prevention of periampullary cancer in FAP, since endoscopic screening for tumors of the proximal small intestine and surgical resection of the duodenum (*e.g.*, the Whipple procedure) are relatively unsatisfactory methods compared to their counterparts in the colon.

Aberrant crypts and ACF have been proposed as possible precursor lesions in carcinogenesis (15). Methylene blue staining in the present experiments indicates that aberrant crypts are extremely rare in *Min*/+ mice (less than 1/colon on average). This is in contrast to the large number of ACF observed in the colon of mice or rats treated with dimethylhydrazine or azoxymethane and may be another reflection of the difference between the "spontaneous" pure adenoma Min model and the carcinogen-induced model. The latter has frequent mutations in the K-*ras* oncogene (19) that may be more closely associated with aberrant crypt formation than *Apc* mutations. Consistent with our observations in mice, in humans with familial polyposis, ACF are not increased, and mutations in *APC* are found much less frequently than K-*ras* mutations in these foci. In the present work, tumors developed spontaneously (no exogenous carcinogen was administered to the Min mice). The observed effect of piroxicam on tumor multiplicity may reflect an influence on tumor growth or initiation, or both. Chemopreventive effects on initiation could be studied in future experiments by inducing additional tumors with a single dose of carcinogen.

We believe that chemoprevention studies with the Min mouse model are clinically relevant since this is a pure adenoma model with a defined genetic etiology that closely mimics the mechanism of *APC* gene inactivation observed in FAP and most sporadic human colon adenomas. The germline mutation in *Min*/+ mice is a heterozygous nonsense mutation in codon 850 of the *Apc* gene, and somatic mutation occurs in adenomas inactivating the normal *Apc* gene function through somatic loss of heterozygosity (frequently, deletion of the entire normal chromosome) (20). The Min mouse model has advantages relative to many carcinogen-induced tumor models since it has a clearly defined and directly relevant genetic lesion. The Min model also has advantages compared to *in vitro* cell culture studies since: (*a*) drug effects and interactions mediated by cell types other than solely the colonic epithelial cell can be detected; (*b*) the *in vivo* model by definition circumvents potential use of biologically irrelevant doses; and (*c*) it is more useful for optimizing chemoprevention targeted at early adenomatous neoplasms than cell culture studies that use transformed tumor cells. The Min mouse model should continue to be useful for testing a variety of chemopreventive agents in the future.

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