R-flurbiprofen suppresses distal nonmucin-producing colorectal tumors in azoxymethane-treated rats, without suppressing eicosanoid production

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Martin JE, Le Leu RR, Hu Y, Young GP. R-flurbiprofen suppresses distal nonmucin-producing colorectal tumors in azoxymethane-treated rats, without suppressing eicosanoid production. Am J Physiol Gastrointest Liver Physiol 298: G860–G864, 2010. First published March 25, 2010; doi:10.1152/ajpgi.00330.2009.—Toxicity and gastrointestinal side effects limits the use of nonsteroidal anti-inflammatory drugs (NSAIDs) as agents to prevent colorectal cancer. These undesirable effects appear to be related to the inhibition of cyclooxygenase-associated pathways. Using the azoxymethane (AOM)-rat model of carcinogenesis, we aimed to test the potency of a low-toxicity R-flurbiprofen and whether NSAIDs have differing effects on regional tumor subtypes. Groups of 50 rats were gavaged 6 days a week with drug. After 1 and 2 wk on drug, rats were given intraperitoneal injection of AOM (15 mg/kg body wt). Groups were controls, sulindac (nonselective cyclooxygenase inhibitor) 5 and 20 mg/kg body wt per day, and R-flurbiprofen 30 mg/kg body wt per day. Tumor incidence of colon tumors was significantly reduced in the sulindac 20 mg (P < 0.001) and the R-flurbiprofen groups (P < 0.03) compared with the control group. The sulindac 20 mg and R-flurbiprofen groups also showed a reduced number of distal colon tumors (P < 0.03), whereas proximal tumors were not affected. Tumors only of the nonmucinous subtype were significantly reduced with the sulindac 20 mg and R-flurbiprofen groups (P < 0.001). Tumor size was not significantly different between all groups. Only the sulindac 20 mg group showed a reduced colonic prostaglandin E2 concentration. The sulindac groups showed a dose-dependant reduction in body weight gain (P < 0.001). In conclusion, R-flurbiprofen at a dose of 30 mg/kg body wt per day was well tolerated by the animals and, along with sulindac at 30 mg/day body wt, showed protection against the development of colon cancer in the rat-AOM model.

In the AOM model there are also distinct histological subtypes of colorectal tumor (29), a fact largely ignored by previous interventional studies. The different subtypes have different regional distributions within the colon (20, 26) and may well arise by different biological pathways. NSAIDs may have different effects on the different tumor subtypes.

In the rat-AOM model (the mouse equivalent of Familial Adenomatous Polyposis) (31). Furthermore, some authors have expressed doubts about its relative potency and concerns about liver toxicity at high dose (28, 32).

R-flurbiprofen is the R enantiomer of the NSAID flurbiprofen, which does not inhibit COX. It has been shown to be effective in the Min mouse, in contrast to sulindac sulfone (31). However, this particular study using the Min mouse model only reported on small intestinal tumors, and no effect on colorectal tumors was reported. Furthermore a major drawback of this animal model of human colon cancer is that their tumors occur predominantly in the small intestine, not the colon. For this reason it is important that the effect on colorectal tumors be examined using an appropriate animal tumor model such as the rat-azoxymethane (AOM) model of colorectal cancer where the majority of tumors arise in the distal colon. AOM-induced tumors share many histopathological characteristics with human tumors. And like human tumors, are often mutated on K-ras and β-catenin genes and show microsatellite instability (5).

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to be protective in both animal and human studies of colorectal cancer (1, 2, 4, 9, 14, 15, 21, 23, 24, 30). However, the major concern about NSAIDs as preventive agents for colorectal cancer remains their toxicity.

The major attribute of NSAIDs responsible for their toxicity is inhibition of cyclooxygenase (COX). There is, however, doubt about whether COX inhibition is responsible for all of their protective effects. A NSAID-like compound, which does not inhibit COX, but does prevent colorectal cancer, may represent a significant advance.

Sulindac sulfone, a metabolite of the NSAID sulindac, has been shown to be protective in both animal and human studies of colorectal cancer (1, 2, 4, 9, 14, 15, 21, 23, 24, 30). However, the major concern about NSAIDs as preventive agents for colorectal cancer remains their toxicity. A NSAID-like compound, which does not inhibit COX, but does prevent colorectal cancer, may represent a significant advance.

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was administered in two doses of 15 mg/kg body wt, 1 wk apart. This was administered subcutaneously and is sufficient to induce colorectal tumors. Drug doses were sulindac at 5 mg/kg body wt per day, sulindac at 20 mg/kg body wt per day, and R-flurbiprofen at 30 mg/kg body wt per day. These doses were chosen on the basis of our previous study using NSAIDs (10). This dose of R-flurbiprofen is known from previous studies to be the maximum tolerated (12), and R-flurbiprofen is effective at inhibiting tumor markers at this dose (14, 17, 23, 24).

Sulindac at the low dose suppresses PGE2 production, without suppressing aberrant crypt foci (ACF) significantly, whereas sulindac at 20 mg/kg body wt per day, and R-flurbiprofen at 30 mg/kg body wt per day. These doses were chosen on the basis of our previous study using NSAIDs (10). This dose of R-flurbiprofen is known from previous studies to be the maximum tolerated (12), and R-flurbiprofen is effective at inhibiting tumor markers at this dose (14, 17, 23, 24).

Monitoring. Rats were reviewed daily. Weight was measured weekly. Rats in poor condition were weighed daily. Rats losing greater than 10% of body weight were euthanized, and an autopsy was performed. Where the cause of death was unclear, the body was examined by a veterinary pathologist. Ten rats died early in the study (before week 9) and at autopsy had signs of AOM toxicity. One died from a bowel obstruction 12 wk into the study. A further 3 rats died from head and neck and small bowel tumors, with no sign of colonic neoplasia. Because these rats died before colonic tumor development could occur, they were excluded from the final analysis.

Tumor analysis. Thirty weeks after AOM treatment, rats were euthanized by CO2-induced narcosis. The colon and small bowel were immediately removed using a dissecting microscope and flushed with ice-cold saline. The stomach, esophagus, and small bowel were examined fresh for any neoplastic lesions. The colon was flattened on Hybond C, fixed in 10% formalin for 18 h, and then stored in 70% ethanol for further analysis. Histological analysis of tumors was performed on sections of 4 microns using hematoxylin and eosin staining. Tumors were classified according to the classification of Sunter et al. (26) with some modification, as mucinous (adenocarcinomas) or nonmucinous in type (adenomatous, carcinomas). The location of the tumors was recorded as either proximal (within the herring bone area of the colon) or distal. Tumor size was measured as the tumor-size index (TSI, see formula below) (17). The TSI was calculated separately for each histological subtype because of the different shapes of each type. The formula for the TSI is as follows:

\[
\text{Tumour size index} = \log_{10} \left[ \sum \frac{(\text{diameter } 1 + \text{diameter } 2)^2}{2} \right]
\]

PGE2 analysis. Three standard colonic biopsies were taken from the distal end of each colon and incubated at 37°C in 2 ml of cell culture medium. After 1 h the medium was collected and frozen at −20°C. Samples were thawed, and 100 μl was mixed with 100 μl radioimmunoassay buffer (0.1% gelatin, 0.9% NaCl, 0.01 M Tris base, and 0.05% Na Azide, pH 7.3) and 100 μl of [3H]-labeled PGE2 (2 μCi diluted in 10 ml of Na2CO3 solution). To this then was added 100 μl of PGE2 antiserum diluted in radioimmunoassay buffer 1:4,000. A sample (0.5 ml) of cold charcoal-dextran was added and vortexed. These samples were incubated at 37°C for 2 h and 4°C for 1 h and then centrifuged for 20 min at 1300 g, and charcoal was removed. Scintillant was added and 3H counted. Concentrations of PGE2 were estimated by comparison with a standard curve generated from known standards. Each assay was performed in triplicate.

Statistical analysis. Statistical analyses were performed using SPSS for Windows, version 11.0 (SPSS, Chicago, IL). The proportion of rats with tumors and tumor distribution were analyzed using Pearson’s Chi-squared analysis. The number of tumors per rat was analyzed using Mann-Whitney U-test. The TSI, weight gain, and PGE2 concentration were analyzed using one-way ANOVA followed by Tukey’s multiple-comparison test. Differences were considered statistically significant at P < 0.05.

RESULTS

Colonic tumors. The effects of the different drug treatments on tumor incidence (proportion of rats that develop a tumor), tumor distribution (proximal or distal), histological subtype (mucinous or nonmucinous tumors), number of tumors per rat, and TSI index are shown in Table 1. The high dose of sulindac reduced the proportion of rats developing colorectal tumors by 60% compared with the control group. (P < 0.001). The R-flurbiprofen treatment reduced the proportion of rats with tumors by 34% compared with the control group (P < 0.03).

Overall more tumors occurred in the distal colon than in the proximal colon. The high-dose sulindac treatment significantly reduced the number of distal tumors compared with the control group (P < 0.001) and the low-dose sulindac group (P < 0.01), whereas the R-flurbiprofen treatment significantly reduced the number of distal colorectal tumors compared with the control group (P < 0.03). There were no significant differences between the drug groups in the numbers of rats with proximal tumors.

In the colon, AOM resulted in two distinct subtypes of tumors; these subtypes have been previously described (8, 20, 26, 29). Nonmucinous tumors (see Fig. 1, C and D) accounted for 65.2% of the tumors found. Mucinous tumors (see Fig. 1, A and B) accounted for the remainder. Nonmucinous tumors

| Table 1. Indices of AOM-induced colon tumors in rats according to drug treatment |
|-------------------------------------------------|----------------|----------------|----------------|
| | Control | Sulindac, 5 mg | Sulindac, 20 mg | R-Flurbiprofen, 30 mg |
| Tumor Incidence | 69% (34/49) | 54% (26/48) | 27% (12/44)* | 46% (21/46)* |
| Tumor Distribution | | | | |
| Proximal Tumors, total number | 6 | 8 | 5 | 7 |
| Distal Tumors, total number | 29 | 20 | 7a | 16b |
| Histological Subtype | | | | |
| Mucinous Tumors, total number | 8 | 9 | 8 | 13 |
| Nonmucinous, total number | 27 | 19 | 4a | 10b |
| Number of Tumors per Rat, multiplicity | 1.0 ± 0.20 | 0.86 ± 0.23 | 0.32 ± 0.13c | 0.66 ± 0.17 |
| Mucinous Tumor Multiplicity | 0.18 ± 0.06 | 0.21 ± 0.07 | 0.23 ± 0.08 | 0.41 ± 0.09 |
| Nonmucinous Tumor Multiplicity | 0.80 ± 0.13 | 0.65 ± 0.16 | 0.09 ± 0.04e | 0.24 ± 0.07f |
| TSI for All Colon Tumors | 1.70 ± 0.20 | 1.86 ± 0.14 | 1.98 ± 0.17 | 1.69 ± 0.20 |
| Mucinous TSI | 1.59 ± 0.31 | 1.61 ± 0.16 | 2.05 ± 0.13 | 1.62 ± 0.18 |
| Nonmucinous TSI | 1.80 ± 0.10 | 2.10 ± 0.12 | 1.91 ± 0.21 | 1.77 ± 0.20 |

Applicable values are means ± SE. Note that some rats had more than one tumor per colon. *P < 0.001 vs. control, aP < 0.05 vs. control, bP < 0.01 vs. sulindac 5 mg, cP < 0.01 vs. sulindac 5 mg (analyzed using Pearson’s χ2 test), dP < 0.001 vs. control, eP < 0.05 vs. sulindac 5 mg, fP < 0.05 vs. sulindac 5 mg (analyzed using Mann-Whitney U-test for 2 independent samples). AOM, azoxymethane; TSI, tumor-size index.
occurred predominantly in the distal colon and were predominantly well differentiated. Nonmucinous tumors were poly-
ploid or spherical macroscopically. Mucinous tumors were found predominantly in the proximal colon and were more poorly differentiated. Mucinous tumors were consistently found closely associated with lymphoid aggregates and had a flattened disc shape. The high-dose sulindac treatment group and R-flurbiprofen treatment group significantly reduced the number of nonmucinous tumors compared with the control group ($P < 0.001$) but had no effect on the mucinous subtype. No significant protective effects were observed with the low-
dose sulindac treatment group.

TSI for both the mucinous and nonmucinous subtypes was not significantly different between control and drug intervention groups.

**Body weight.** The mean final body weight and weight gain of the different treatment groups over the 30-wk period are shown in Table 2. The R-flurbiprofen drug treatment was well tolerated and resulted in similar amounts of weight gain compared with the control group. In contrast the rats treated with sulindac showed a significant dose-dependent reduction in final body weight and reduced weight gain compared with the control group.

**PGE2 production.** The PGE2 levels (ng/100 ml) in the colon are shown in Table 2. The high-dose sulindac treatment had significantly reduced levels compared with the R-flurbiprofen treatment ($P < 0.05$) and the control group ($PP < 0.001$). The R-flurbiprofen treatment did not reduce PGE2 production.

**DISCUSSION**

This study shows for the first time that R-flurbiprofen is effective at preventing colorectal cancer in a carcinogen-in-
duced animal model. This was achieved without significant inhibition of PGE2 production, as this enantiomer does not inhibit COX, indicating that effective chemoprevention can be achieved without COX inhibition. No toxicity was apparent in the R-flurbiprofen treatment, as the animals gained the same amount of weight as controls. Thus R-flurbiprofen appears to be an effective chemopreventive agent with no obvious signs of toxicity.

When analyzed by regional distribution, only distal tumors were reduced by the R-flurbiprofen and the high dose of sulindac. Proximal tumors were not significantly affected. This is probably accounted for by the effect on the different histo-

![Fig. 1. Azoxymethane (AOM)-induced mucinous and nonmucinous adenocarcinoma in rat colon. A: mucinous adenocarcinoma (×40 magnification). B: mucinous adenocarcinoma (×200 magnification). C: nonmucinous adenocarcinoma (×40 magnification). D: nonmucinous adenocarcinoma (×200 magnification).](image-url)

Table 2. **Final body weights, weight gain per 30 wk, and colonic PGE2 production**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sulindac, 5 mg</th>
<th>Sulindac, 20 mg</th>
<th>R-Flurbiprofen, 30 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Body Weight, g</td>
<td>732 ± 11</td>
<td>671 ± 13&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>577 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>722 ± 10</td>
</tr>
<tr>
<td>Body Weight Gain, g over 30 wk</td>
<td>391 ± 12</td>
<td>326 ± 12&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>235 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>374 ± 11</td>
</tr>
<tr>
<td>PGE2, ng/100 ml</td>
<td>1.76 ± 0.18</td>
<td>1.44 ± 0.16</td>
<td>1.14 ± 0.12&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>1.62 ± 0.14</td>
</tr>
</tbody>
</table>

Values are means ± SE. <sup>a</sup>$P < 0.001$ vs. control, <sup>b</sup>$P < 0.001$ vs. R-flurbiprofen 30 mg, <sup>P</sup>$P < 0.01$ vs. R-flurbiprofen 30 mg, <sup>d</sup>$P < 0.01$ vs. control, <sup>e</sup>$P < 0.05$ vs. R-flurbiprofen 30 mg (ANOVA with Tukey’s multiple-comparison test).
logical subtypes, which are distributed differently. Nonmucinous tumors are mostly distally distributed and were significantly reduced by both sulindac and R-flurbiprofen. Mucinous tumors, in contrast, are mostly proximally distributed and were unaffected by either. In previous studies it has been noted that nonmucinous tumors correlate with ACF numbers (20) and appear to follow an adenoma-carcinoma sequence similar to human sporadic colonic carcinoma. Mucinous tumors, in contrast, are associated with lymphoid aggregates, do not correlate with ACF numbers, and appear to arise de novo (20). This implies that the genesis of these two histological subtypes occurs by different biological pathways (29); it is thus not surprising that NSAIDs have different effects on them. No previous study has reported a differential effect of NSAIDs or NSAID-like compounds on different histological subtypes of tumors although one study has reported a different effect on tumors in different regions of the colon. Liu et al. (18) reported that piroxicam was more effective in reducing the incidence of proximal tumors. In the present study, there was a marked inhibition of distal tumors but no significant inhibition of proximal tumors. These results appear contradictory, but, as Liu et al. (18) did not report the histological subtypes of the tumors, it is not possible to know whether subtype, and not location, explains the difference or whether it is due to a fundamentally different action by piroxicam.

Neither R-flurbiprofen nor sulindac affected the TSI. Sulindac has been shown previously to reduce tumor growth when begun after tumor development (4). A possible explanation for why we did not see a reduction in tumor size in the present study was that these drugs may have their inhibitory effects on AOM-induced oncogenesis at the very stages of DNA damage (11), by removing the DNA-damaged cells that may progress further to tumors.

There was a trend to a greater suppressive effect with sulindac than R-flurbiprofen. Given the dose-response effect demonstrated with sulindac, this might simply reflect the dose of R-flurbiprofen that was chosen in that it was submaximal. A higher dose in rodents might not be feasible. Rats and mice show some inversion of R-flurbiprofen to its S-enantiomer (30). The COX-related side effects of the S-enantiomer limits the maximal tolerated dose in this model. In contrast, no measurable inversion occurs in humans at low doses (50–100 mg) (7), whereas higher doses of the R enantiomer appear to be well tolerated with no side effects seen with doses of 1,600 mg/day (6). Higher relative doses may therefore be feasible in humans, and the relative potencies of these drugs will need further consideration.

NSAIDs such as sulindac have shown promising tumor chemoprevention (16, 19) especially through their anti-inflammatory properties although toxicity from COX inhibition and the suppression of prostaglandin synthesis limit their use for chemoprevention (25, 28). In the present study, we reported that the high dose of sulindac (20 mg/kg body wt per day) was the only drug treatment that significantly reduced colonic PGE2 levels; furthermore this treatment also showed a detrimental effect on the growth of the rats. Conversely, the R-flurbiprofen treatment showed no reduction in PGE2 levels, and furthermore there was no adverse effect on the body weights of these rats. This indicates that R-flurbiprofen is well tolerated and that its underlying effects on tumor suppression may be independent of COX inhibition. We did not observe any other signs of gastrointestinal toxicity in any of the drug treatments.

The molecular mechanisms underlying the protective effects of R-flurbiprofen are not fully understood but are likely to involve induction of apoptosis. We have previously demonstrated that R-Flurbiprofen at the same dosage used in the present study is able to significantly enhance the apoptotic response to the colon carcinogen AOM at the time of initiation of DNA damage (12). Furthermore, we previously demonstrated that sulindac was proapoptotic and reduces the colonic mutational load resulting from the carcinogen by eliminating DNA-damaged cells (11). It is feasible that the mechanism of protection against colon cancer by R-flurbiprofen may be through the removal of DNA-damaged cells at the time of initiation of DNA damage. More work is now needed to further determine any other mechanisms that underlie the protective effect of R-flurbiprofen.

The interventions chosen in the present study are ones that are achievable in humans. R-flurbiprofen in a recent human intervention study in healthy individuals (6) has been shown to be safe and well tolerated in a range of doses (400, 800, and 1600 mg/day). In the present study, the 30 mg/kg body wt dose of R-flurbiprofen to rats would be comparable to the high dose used in the human intervention study (6). Sulindac in humans is generally recommended at a maximum level of 400 mg/day; this dose would equate to the low-dose sulindac (5 mg/kg body wt per day) in our rat study, whereas the high-dose sulindac (20 mg/kg body wt per day) also used in our rat study may not be achievable in humans with out the increased risk of toxicity (27).

In summary, R-flurbiprofen (a non-COX inhibitor) demonstrated comparable suppressive effects against AOM-induced colorectal cancer in rats to that of the high-dose sulindac (nonselective COX inhibitor). The R-flurbiprofen treatment appeared to act independent of COX inhibition and was less toxic than the sulindac treatments in terms of body weight loss. These results indicate that further evaluation of R-flurbiprofen as a chemopreventive agent for colorectal cancer in humans is justified.

GRANTS

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DISCLOSURES

The authors declare no potential conflicts of interest.

REFERENCES

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R-FLURBIPROFEN SUPPRESSES DISTAL NONMUCIN-PRODUCING COLORECTAL TUMORS


