Comparing the Effects of COX and Non-COX-inhibiting NSAIDs on Enhancement of Apoptosis and Inhibition of Aberrant Crypt Foci Formation in a Rat Colorectal Cancer Model

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Abstract. The protection against colorectal cancer (CRC) by non-steroidal anti-inflammatory drugs (NSAIDs) is in part dependent on inhibition of cyclooxygenase (COX). We compared the efficacy of the non-COX-inhibiting R-flurbiprofen (R-FB) with COX-inhibiting sulindac and racemic flurbiprofen (Rac-FB), and determined their effects on apoptosis, in an azoxymethane (AOM)-induced rat CRC model. In experiment 1, groups of rats were given a daily drug gavage (R-FB 30 mg/kg, Rac-FB 10 mg/kg and Sulindac 20 mg/kg) for one week, followed by AOM treatment and were sacrificed eight hours later, colons were examined for apoptosis and cell proliferation. In experiment 2, groups of rats were given two AOM treatments, followed by a daily drug gavage until they were sacrificed ten weeks later, and colons were examined for apoptosis and cell proliferation. All drugs significantly enhanced apoptosis and inhibited ACF, irrespective of their COX-inhibiting potency (p<0.01), but sulindac was more potent in inhibition of large ACF, p<0.05. COX-inhibiting sulindac achieved the greatest protective effect. The greater safety profile of Rac-FB should provide an advantage for chemoprevention.

Colorectal Cancer (CRC) is one of the leading causes of cancer death worldwide (1). A large body of clinical studies provide strong evidence, indicating that the use of non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the risk of CRC. Experimental studies have demonstrated that NSAIDs reduce the incidence of carcinogen-induced colon tumours in rodents, and several epidemiological investigations have also shown a 40-50% reduction in the risk of colorectal adenoma and cancer in individuals receiving NSAIDs (2, 3). Moreover, patients with familial adenomatous polyposis taking sulindac or other NSAIDs experience a reduction in adenoma formation (4). The chemopreventive effects of NSAIDs are, at least, in part related to inhibition of cyclooxygenase-2 (COX2), the inducible isoform of COX that catalyses the conversion of arachidonic acid to prostaglandin. Approximately 50% of adenomas and 80% of CRC express high levels of COX2 mRNA and protein in neoplastic tissue (5). However, COX inhibition in the context of CRC chemoprevention may also be responsible for NSAIDs toxicity. In fact, the toxicity is one of the major barriers to wider use of NSAIDs in CRC prevention. There is growing evidence that a COX-independent mechanism may be, at least partially, responsible for the chemopreventive properties of NSAIDs (6-10). NSAIDs which do not inhibit COX but still are effective in CRC prevention would represent a significant advance in prevention or treatment of CRC, with potential for reduced toxicity. The molecular mechanisms of action of COX-independent NSAIDs and the timing of their action are not fully explained (11). Studies are therefore needed to compare the efficacy of non-COX-inhibiting NSAIDs with COX-inhibiting ones in order to understand the mechanism and timing of their action.

Studies in animal models have shown that non-COX-inhibiting NSAIDs may be useful in primary prevention of CRC. Sulindac sulfone (a metabolite of the parent Sulindac) inhibits tumourigenesis in the azoxymethane (AOM)-induced rat CRC model without suppressing prostaglandin levels (12). Another potential candidate is the non-COX-inhibiting R enantiomer of the NSAID flurbiprofen (13). Flurbiprofen is a member of 2-aryl propionic acid group of NSAIDs and...
consists of two enantiomers, of which \( S \) flurbiprofen (S-FB) is a potent COX inhibitor whereas the \( R \) enantiomer (R-FB) is a much less potent inhibitor of COX. R-FB has been shown to be protective against intestinal tumourigenesis in the \( Apc^{Min+} \) mice (14, 15). But there is about 15-24% bioinversion of R-FB to S-FB in mice, and approximately 4% in rats, whereas in humans R-FB undergoes very little bioinversion to S-FB (16), suggesting the rat has a bioinversion profile sufficiently similar to humans and which might be useful for pre-clinical studies.

Increased apoptosis and reduced cell proliferation have been demonstrated for various NSAIDs. Some NSAIDs are known to be pro-apoptotic in various settings (17). For example, COX-inhibiting sulindac is known to inhibit colorectal tumourigenesis by inducing apoptosis of cells which have already undergone neoplastic transformation in CRC in vivo (18-20). Non-COX-inhibiting NSAIDs also induce apoptosis of human colon carcinoma cells (2, 21-23). However, the effective chemoprevention of colon cancer by NSAIDs lies in the elimination of DNA-damaged cells that are inappropriately activated by oncogenic events through the induction of apoptosis. Hence it is important to examine if NSAIDs might facilitate apoptotic deletion of DNA-damaged cells at the time of initiation (before neoplastic transformation has occurred) in the AOM CRC model, and if enhanced apoptosis is associated with suppression of early pre-neoplastic lesions, formation of aberrant crypt foci (ACF). The present study was designed to determine the effects of non-COX-inhibiting R-FB on early apoptotic response to initiation of oncogenesis and ACF formation in an AOM rat model, and compare its safety and efficacy with two COX-inhibiting NSAIDs: sulindac and racemic-flurbiprofen (Rac-FB, a 50/50 mixture of \( R \) and S-FB).

Materials and Methods

Chemicals. AOM was purchased from Sigma Chemical (St. Louis, MO, USA); sulindac was supplied by Merck Pty. Ltd (Victoria, Australia); and Flurbiprofen was a gift from Encore Pharmaceuticals, CA, USA (Dr. W. Wechter).

Animals. A total of 108 male Sprague-Dawley rats were obtained from the Animal Resource Center, Adelaide University, Australia. The study was approved by the Animal Welfare Committee at Flinders University (#469/98). Two experiments were performed, using 48 rats for a short-term experiment and 60 rats for a long-term experiment. For each experiment, animals were divided randomly into four equal experimental groups (with comparable initial body weights), housed in plastic cages (three per cage) and maintained in a temperature- and humidity-controlled animal facility with a 12-h light/dark cycle at 22±2°C and 80±10% humidity. Animals were given free access to water. Animals were weighed weekly and were monitored daily for clinical signs of ill health throughout the study.

Diets. The experimental diet (Table I) fed to the animals was based on a modified American Institute of Nutrition (AIN)-76A diet for rodents (24). The diet had a protein:carbohydrate:fat balance of 20:55:20 by weight.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Modified AIN diet‡ (g/100g diet)</th>
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<tbody>
<tr>
<td>Casein</td>
<td>20</td>
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<td>Sucrose</td>
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<tr>
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<tr>
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<tr>
<td>Methionine</td>
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Drugs. The drug groups were: R-FB at 30 mg/kg; Rac-FB at 10 mg/kg and sulindac at 20 mg/kg. R-FB at 30 mg/kg is the maximal tolerated dose (W. Wechter, Loma Linda CA, USA, personal communication); the 4% bio-inversion of R-FB to S-FB in vivo (equivalent to 2.4 mg/kg of Rac-FB) production, and 10 mg/kg also represents a maximal tolerated dose.

Short-term study. Beginning at eight weeks of age, groups of rats (12/group) were fed a modified AIN diet for two weeks, followed by one week of drug daily (i.e. R-FB at 30 mg/kg, Rac-FB at 10 mg/kg and sulindac at 20 mg/kg) via gavage. A group without drug treatment was used as control. At the end of drug treatments, a single subcutaneous injection of AOM was given at dose of 10 mg/kg to induce DNA damage and acute apoptotic response. Animals were euthanized by CO2 eight hours later, this being the time of maximal apoptotic response to AOM in the rat (25). Two centimeters of distal colon were removed, placed in 10% paraformaldehyde and embedded in paraffin for study of apoptosis and cell proliferation (Figure 1A).

Long-term ACF study. Beginning at five weeks of age, groups of rats (15/group) were fed a modified AIN diet for two weeks, then received two consecutive weekly subcutaneous injections of AOM (15 mg/kg body weight) to induce colonic oncogenesis. After the second AOM injection, rats were given drug daily (i.e. R-FB at...
30 mg/kg, Rac-FB at 10 mg/kg and sulindac at 20 mg/kg) via gavage. Rats remained on the same diet throughout the study until killed by CO₂ asphyxiation ten weeks after drug treatment. A group without drug treatment was used as control. On sacrifice, the entire colon was rapidly removed, cleaned and fixed flat on Hi-bond C protein binding paper (GE Healthcare, VWR International, Victoria, Australia) and finally fixed in 10% paraformaldehyde. These flat segments of colon were then stored in 70% ethanol until evaluation for ACF. Three standard colonic biopsies were also taken from each colon for PGE₂ analysis (Figure 1B).

Measurement of apoptosis. Paraffin-embedded sections (4 μm) were stained with hematoxylin and evaluated for the frequency of colonic epithelial cells undergoing apoptosis as previously described in detail and validated relative to Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) staining (25). Twenty colonic crypts were chosen for counting for each rat by an independent observer unaware of the drug treatment on the basis that they had a lumen and an intact single row of epithelial cells on each side. Apoptotic cells were identified by cell shrinkage with a halo separating them from surrounding cells, nuclear condensation and formation of apoptotic bodies. Apoptosis was quantified as the mean number of apoptotic cells per crypt.

Measurement of cell proliferation. The proliferative activity of colon epithelial cells was measured using immunohistochemical staining with a monoclonal antibody to Proliferating Cell Nuclear Antigen (PCNA) (PC-10, Santa Cruz, USA). In brief, the sections were treated with 0.3% hydrogen peroxide for 20 min to reduce the endogenous peroxidase activity. Sections were blocked by 10% normal horse serum for 30 mins to block non-specific staining. PC-10 clone PCNA was applied at 1:400 dilution in 10% normal horse serum overnight at room temperature. The sections then were incubated with biotinylated secondary rabbit anti-mouse IgG (Pharmingen, San Diego, CA, USA) for 30 min and Vectasin ABC reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min. Slides were visualized by incubating with 3,3’-diaminobenzene (DAB) substrate and counter-stained with hematoxylin. The expression of PCNA was identified by cell nuclei that stained brown with antibody against PCNA. Proliferation was quantified as the mean number of PCNA positive cells per crypt.

Quantification of ACF. The colons were stained with 0.1% methylene blue and the number of ACF was counted by examination at 40× magnification using a dissecting microscope. ACF was identified by increased size, elevated appearance from the surrounding mucosa and slit-like shape of the luminal opening and
scored without knowledge of the dietary treatment. The total number of ACF per colon was recorded from the distal to proximal end by a single-blind method, then the average number of ACF of the colon, with no discrimination of the multiplicity, was determined as the mean of total ACF/length of section (ACF density/cm). The number of aberrant crypts with large crypts (>3 crypt/focus) was also counted.

**PGE 2 analyses.** PGE 2 was measured by radioimmunoassay (RIA) as previously described (26). In brief, three standard colonic biopsies were taken from the distal end of each colon and incubated at 37˚C in cell culture medium. After 1 h the medium was collected, and 100 μl sample was mixed with 100 μl RIA buffer and 10 μl of tritium [3H]-labelled PGE 2 (2 μCi diluted in 10 ml of Na 2CO3 solution). To this were added 100 μl of PGE 2 antisera diluted in RIA bufferation. Samples were then incubated at 37˚C for two hours and 4˚C for one hour, followed by centrifugation (15,000 ×g) for 20 min. Scintillant was added and [3H] counted. Concentrations of PGE 2 were estimated by comparison with a standard curve generated from known standards (26). Each assay was performed in triplicate.

**Statistical analysis.** Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Apoptosis, PCNA, PGE 2 and weight gains were analyzed using one-way ANOVA with correction for multiple comparisons by Tukey’s post-hoc test. Between-group comparisons of ACF counts were assessed using logistic and Poisson regression respectively with simple linear contrasts. Differences between groups were considered significant when p<0.05.

### Results

**General observation.** The mean final body weight and weight gain of the rats under different drug treatments over the fourteen-week period are shown in Table II. The R-FB drug treatment was well-tolerated and led to a weight gain comparable to that of the control group. In contrast, rats treated with sulindac (p<0.001) had a significant reduction in weight gain, compared with the control group. While rats treated with Rac-FB also had significantly lower body weight (p<0.01), the weight loss was not as severe as that of rats treated with sulindac. Nevertheless, all rats survived until the termination of the ACF experiment; examination of the small intestine, liver and kidney did not reveal any abnormalities.

**Effects of NSAIDs on apoptosis.** All three drugs significantly enhanced the acute apoptotic response to carcinogen-induced DNA damage irrespective of their COX-inhibiting potency compared with the control group p<0.01 (Figure 2A). Apoptosis was mostly located in the lower compartment of the crypts, where cells including stem cells form O6-methylguanine (O 6meG) adduct (25). There was no difference between COX-inhibiting sulindac and Rac-FB, and non-COX-inhibiting R-FB.
Effects of NSAIDs on cell proliferation. None of the drugs affected the average number of cells per crypt, nor cell proliferation in the distal colon when expressed as number of the PCNA-positive cells per crypt (Figure 2B and 2C).

Effects of NSAIDs on ACF formation. ACF were observed predominantly in the distal colon. Results are shown in Table III for total ACF, ACF density and crypt multiplicity (large ACF). The total number of ACF was significantly reduced by all three drugs \((p<0.01)\), compared with the control, but sulindac was more potent than both R-FB and Rac-FB \((p<0.05)\). Significant reduction was also observed in ACF density for all three drugs compared with the control group \((p<0.01)\). However, the numbers of large ACF (with four crypts per focus or more) were significantly reduced by Rac-FB \((p<0.05)\) and sulindac \((p<0.01)\), but not by R-FB. Sulindac was also more effective in reducing large ACF (the most relevant for predicting subsequent invasive tumour lesions) than Rac-FB \((p<0.05)\).

Effects of NSAIDs on mucosal PGE 2. The PGE 2 levels in the colon are shown in Table I. PGE 2 production was significantly reduced by Rac-FB and sulindac when compared to the control group \((p<0.01)\), but R-FB did not significantly affect PGE 2 production.

Discussion

Some of the protective actions of NSAIDs against CRC appear to be independent of COX inhibition. It remains to be clarified whether COX-independent mechanisms are effective in cancer prevention. R-FB does not inhibit COX and thus provides the opportunity to directly compare the potency of different protective mechanisms in CRC prevention. In the present study, we used an AOM rat model to compare the effect of R-FB with COX-inhibiting NSAIDs sulindac and Rac-FB on the acute apoptotic response to initiation of oncogenesis and early preneoplastic lesions, and formation of ACF. Our data suggest that all three drugs significantly enhanced the acute apoptotic response to AOM-induced DNA damage irrespective of their COX-inhibiting potency. All drugs significantly reduced the formation of ACF (total ACF and ACF density), but the number of large ACF was significantly reduced only by COX-inhibiting NSAIDs sulindac and Rac-FB, with sulindac showing the greatest protective effect, but this was accompanied by significant weight loss. Our study suggests that the enhanced apoptosis during the early stage of tumor initiation is, in part, responsible for the protection against AOM by NSAIDs, but COX inhibition appears to be necessary for better protection when using ACF as a surrogate biomarker for chemoprevention (27).

Sulindac has been shown to be effective in suppressing ACF at a dose of 20 mg/kg/day in the AOM model and at this dose it induces toxicity (28, 29). Thus, sulindac provides a useful comparison of toxicity and efficacy with which to compare R-FB and Rac-FB. Sulindac had potent inhibitory effect on all measures of ACF compared with R-FB and Rac-FB. In particular, sulindac significantly inhibited the formation of large ACF, which is considered to be most
relevant for predicting subsequent invasive tumorous lesions and is better correlated with the tumour incidence (30). However, it also significantly reduced PGE 2 production, with significant weight loss, thus the toxicity of sulindac may limit its use for chemoprevention in humans. The reason for using Rac-FB for comparison is that Rac-FB contains 50% S-FB (COX-inhibiting) and 50% R-FB (non-COXinhibiting), thus it provides a reasonable comparison to determine the importance of COX inhibition. When comparing the effects of R-FB and Rac-FB on ACF, Rac-FB appeared to be more effective at reducing large ACF than R-FB, although it also included a certain degree of toxicity judged by less weight gain (due to the COX-inhibitory properties of S-FB). In this regard, Rac-FB appears to have advantages over sulindac in terms of safety and public health implications. Given the fact that there is no measurable bio-inversion in humans (31), it is likely that the safety of Rac-FB may be further improved in humans. While R-FB is not as potent as sulindac and Rac-FB in colon cancer inhibition, R-FB appears to have a continuous effect throughout the process of colonic oncogenesis (ACF - adenoma - carcinoma sequence) because its inhibitory effect on ACF parallels its anticancer effect using colon tumour as an endpoint in our previous rat study (26). A dose of 30 mg/kg R-FB is achievable in humans, because this dose in rats would be comparable to the safe and well-tolerated high dose of R-FB (1600 mg/kg) in humans (32). Sulindac in humans is generally recommended at a maximum level of 400 mg/day, which would be equivalent to 5 mg/kg in the rat. At this dose, our preliminary data showed that sulindac did not affect ACF formation (unpublished data), whereas sulindac at 20 mg/kg increased the risk of COX inhibition-related toxicity (28, 29). In APCMin/+ mice treated with sulindac, tumour number and size does not correlate with PGE 2 levels, and so even with COX-inhibiting NSAIDs, their chemopreventive effect may not be solely due to COX inhibition (33). However, the relative potency of these NSAIDs should be cautiously interpreted in animal studies because the relative toxicity and therefore the safe dose are species-dependent.

Transformation of colorectal epithelia from ACF to adenoma and adenocarcinoma is associated with a progressive inhibition of apoptosis and increased cell proliferation (34). NSAIDs are powerful chemopreventive agents for CRC, but the stage at which NSAIDs act to protect has been debated and one group suggests that multiple stages are affected with differing dependence on COX inhibition (35). Enhancement of apoptosis is an important mechanism for the chemopreventive action of NSAIDs. To date, most evidence for NSAIDs on regulating apoptosis has been studied in colorectal tumours or colorectal tumour cells (18-20, 36). In pre-clinical studies, NSAIDs are generally administered during the post-initiation and progression stages and can act relatively late in tumourigenesis to bring about regression of established tumours by inducing apoptosis in an APCMin/+ mouse model and in rat carcinogen-induced CRC model (12, 20, 37, 38). In fact, the chemopreventive efficacy of sulindac sulfone depends on the time of its administration during the carcinogenic process (2): it increases apoptosis when administered during the initiation or post-initiation periods, whereas it has minimal effects on apoptosis when administered during the promotion/progression stage (2, 39). However, the possibility that NSAIDs might regulate an apoptotic elimination of DNA-damaged cells at the time of tumour initiation has not been well-characterized. This early apoptosis in response to genotoxic insult may be a critical time in tumourigenesis, when damaged cells can be eliminated and therefore tumour initiation prevented.

One study has shown that NSAIDs suppressed tumour incidence by enhancing apoptosis of carcinogen-initiated cells (40). Our data in the present study suggest that NSAIDs might exert their chemopreventive effect, in part, by enhancing acute apoptosis. A study in our laboratory has shown that the time course of induction of apoptosis in the colon by AOM (0-72 h) parallels O6-MeG adduct formation (Nyskohus L, unpublished data). In the gastrointestinal tract, an enhanced apoptotic response is an important protective process eliminating cells with DNA damage that may otherwise progress to malignancy (25, 41). Most importantly, this apoptotic response is a regulatory target for a variety of dietary agents and drugs (41-47). Thus NSAIDs may exert a significant pro-apoptotic effect early in oncogenesis which protects against persistence of mutations (K-RAS) and progression to ACF and eventually cancer (48). Up-regulation of caspase-3 and down-regulation of B-cell lymphoma-2 (Bcl-2) have been reported to be associated with this early apoptotic response during tumour initiation (42, 47). These data together with our previous data suggest that apoptotic elimination of DNA-damaged cells could explain the reduced ACF (current study) and tumour formation in rats fed two categories of NSAIDs (26). Another mechanism for the action of NSAIDs may be through inhibition of proliferation (28). While cell proliferation was not affected by all drugs during a short-term study, the inhibition of ACF during a long-term study would suggest the impact of drugs on cell proliferation.

In conclusion, the effect of NSAIDs on enhancing the acute apoptotic response to the initiation of carcinogenesis is irrespective of their COX-inhibiting potency, but a COX-inhibitory effect is necessary for the best chemopreventive effect, because COX-inhibiting sulindac achieved the greatest protective effect against ACF formation. The greater safety profile of Rac-FB should provide with an advantage for its use in chemoprevention.
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References


