



Archived at the Flinders Academic Commons:

<http://dspace.flinders.edu.au/dspace/>

This is a copy of an article published in *Anticancer Research*, and is available online at: <http://ar.iijournals.org/>

Please cite this as: Martin, J.E., Young, G.P., Le Leu, R.K. and Hu, Y., 2013. 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. *Anticancer Research*, 33, Accepted for publication 17 July 2013.

© 2013 International Institute of Anticancer Research.

Paper reproduced here with permission from the publisher.

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

JONATHAN E. MARTIN¹, GRAEME P. YOUNG², RICHARD K. LE LEU² and YING HU²

¹Investigational Procedure Unit, Repatriation General Hospital, Daw Park, South Australia, Australia;

²Flinders Centre for Innovation in Cancer, Flinders University, South Australia, Australia

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 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 killed eight hours later, colons were examined for apoptosis and cell proliferation. In experiment 2, groups of rats were given two AOM treatments, followed by daily drug gavage until they were killed ten weeks later, colons were examined for aberrant crypt foci (ACF) and prostaglandin E 2 production. 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 indicates 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 taking 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)

Correspondence to: Ying Hu, Flinders Centre for Innovation in Cancer, GPO Box 2100, Adelaide, SA 5001, Australia. Tel: +61 822045170, Fax: +618 82045703, e-mail: ying.hu@flinders.edu.au

Key Words: NSAIDs, colon cancer prevention, COX inhibition, apoptosis, ACF, rat model.

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 tumorigenesis in the *APC^{Min/+}* mouse (14, 15). But there is about 15-24% bio-inversion of R-FB to S-FB in mice, and approximately 4% in rats, whereas in humans R-FB undergoes very little bio-inversion to S-FB (16), suggesting the rat has a bio-inversion profile sufficiently similar to humans and which might be useful for preclinical 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 tumorigenesis 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 preneoplastic 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 Centre, 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 12h 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

Table I. *Composition of experimental diets.*

Ingredient	Modified AIN diet [#] (g/100g diet)
Casein	20
Sucrose	20
Olive oil	19
Corn oil	1
Fiber (alpha cell)	5
Corn starch	30
Choline	0.2
Mineral mix‡	3.5
Vitamin mix	1
Methionine	0.3

[#]AIN: a modified American Institute of Nutrition (AIN) -76A diet achieved that had a protein: carbohydrate:fat balance of 20:55:20 by weight.

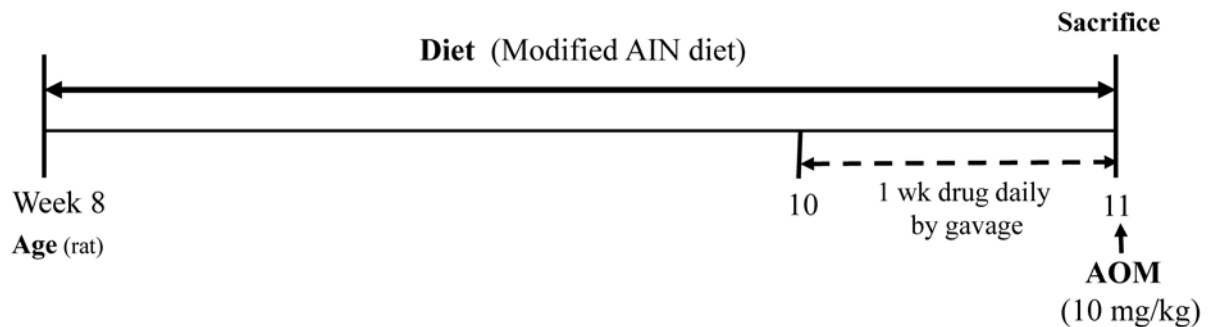
(24). The diet had a protein:carbohydrate:fat balance of 20:55:20 by weight so as to humanize the fat contribution to energy intake to 35%. The fat component was 19% olive oil and 1% corn oil. Olive oil was chosen because it has no effect on eicosanoid production and is not expected to interact with the NSAIDs. A small amount of corn oil was used to provide essential fatty acids. Diet was prepared fresh at 4-weekly intervals, pelleted and stored at -20°C until use.

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 suggests a dose of 30 mg/kg would be converted to around 1.2 mg/kg of S-FB *in vivo* (equivalent to 2.4 mg/kg of Rac-FB). Rac-FB at 10 mg/kg was chosen because our preliminary study showed that 5 mg/kg of Rac-FB inhibits prostaglandin E₂ (PGE₂) production, and 10 mg/kg also represents a maximal tolerated dose. The use of two enantiomers of FB in the studies provides a powerful way to clarify the importance of the COX inhibitory mechanism. Sulindac at 20 mg/kg was chosen because this is its chemoprotective dose, showing colon tumour inhibition in a rat CRC model (18).

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 was 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

A. Short-term experiment



B. Long-term ACF experiment

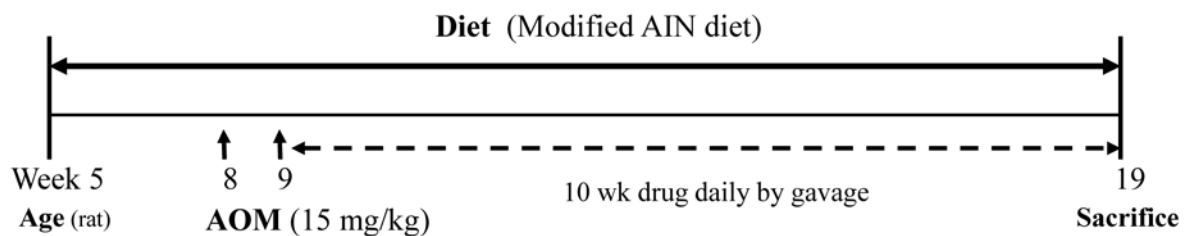


Figure 1. Experimental design for short-term (A) and long-term (B) study (ACF) in an (AOM) -induced rat (CRC) model. A: Short-term study: groups of rats ($n=12$) were given drug daily by gavage at the age of eight weeks. After one week's drug treatment, rats were injected with AOM (10 mg/kg) and killed eight hours later. Colons were examined for apoptosis, cell proliferation and crypt height. B: Long-term ACF study: groups of rats ($n=15$) were given two weekly AOM treatments (15 mg/kg) followed by drug daily by gavage. Rats were killed ten weeks after AOM treatment and colons were evaluated for ACF formation and PGE 2 production.

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 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 μ m) were stained with haematoxylin 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 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 Vectastin ABC reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min. Slides were visualized by incubating with 3,3'-diaminobenzamine (DAB) substrate and counter stained with haematoxylin. The expression of PCNA was identified by cell nuclei that stained brown with antibody to 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 \times 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

Table II. Final body weights and weight gain during 14 weeks of the long-term (ACF) study and colonic (PGE 2) production.

	Final body weight (g)	Body weight gain (g)	PGE 2 production (ng/100 ml)
Control	705±10 ^a	374±15 ^a	2.5±0.4 ^a
R-FB 30 mg/kg	698±12 ^a	364±11 ^a	2.0±0.4 ^a
Rac-FB 10 mg/kg	654±10 ^b	294±8 ^b	0.5±0.4 ^b
Sulindac 20 mg/kg	531±11 ^c	189±10 ^c	0.45±0.4 ^b

R-FB: R flurbiprofen; S-FB: S flurbiprofen; Rac-FB: a 50/50 mixture of R-FB and S-FB. Values are means±SEM. Values in each column with different superscripts are statistically different ($p<0.05$).

Table III. Effects of drugs on the formation of (AOM) -induced (ACF) in rats.

	Total ACF/rat [#]	Large ACF/rat [†]	ACF/cm [‡]
Control	220±30 ^a	31±4.5 ^a	15±2.0 ^a
R-FB 30 mg/kg	152±10 ^b	38±4.5 ^a	9.3±1.6 ^b
Rac-FB 10 mg/kg	153±16 ^b	13.5±2.6 ^b	10.4±1.5 ^b
Sulindac 20 mg/kg	84±23 ^c	6.7±3.6 ^c	5.5±1.0 ^c

R-FB: R flurbiprofen; S-FB: S flurbiprofen; Rac-FB: a 50/50 mixture of R-FB and S-FB. [#]Total number of ACF was calculated as the sum of the small and large ACF. [†]Large ACF was classified by the number of crypts per focus (≥ 4). [‡]The mean of total ACF/length of section (ACF density/cm). Values are means±SEM. Values with different superscripts in each column are statistically different ($p<0.05$).

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 hour 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 2CO 3 solution). To this then was added 100 µl of PGE 2 antisera diluted in RIA buffer. Samples were then incubated at 37°C for two hours and 4°C for one hour, followed by centrifuging (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 analysed 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 abnormality.

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 (O6meG) 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 cell 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 this 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, formation of ACF. Our data suggest 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 ACF 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 it

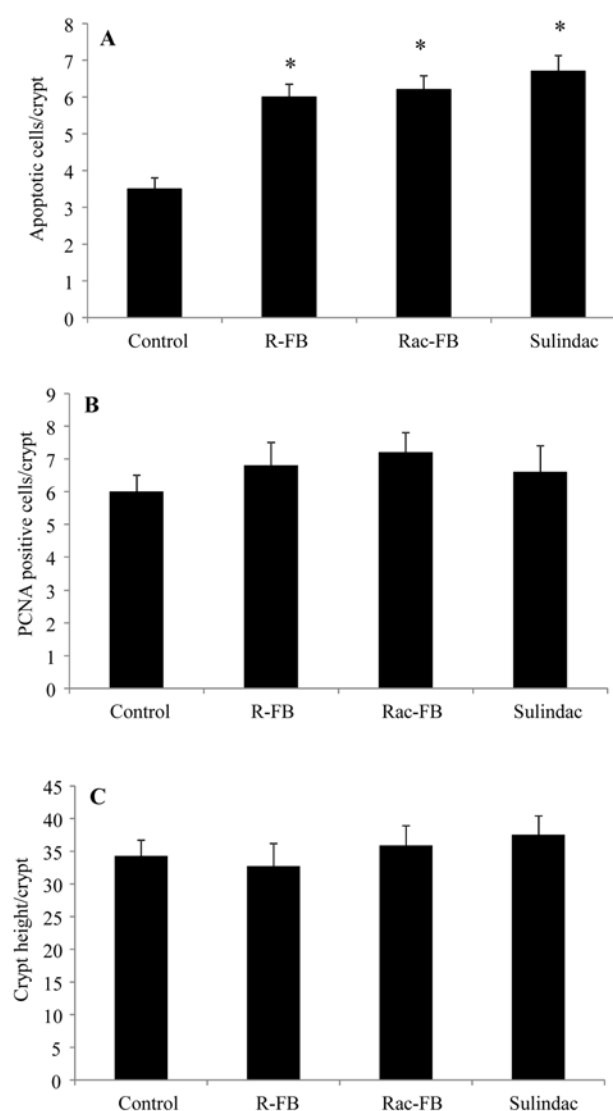


Figure 2. Effects of drugs on apoptosis, cell proliferation and crypt height. Apoptotic cells (A), (PCNA)-stained cells (B) and crypt height (C) of the colonic epithelium of rats for control, 30 mg/kg (R-FB), 10 mg/kg (Rac-FB) and 20 mg/kg sulindac-treated rats eight hours after (AOM) injection. Statistical significance of drug treatment between the groups was analysed by ANOVA ($*p < 0.01$, compared with control group). Data are the mean \pm SEM.

induces toxicity at this dose (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-COX -inhibiting), 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 include 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 there is no measurable bio-inversion in humans (31), it is likely 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 anti - cancer effect using colon tumour as endpoint in our previous rat study (26). A dose of 30 mg/kg R-FB is achievable in human, 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 equivalent to 5 mg/kg in the rat. At this dose, our preliminary data showed 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 *APC^{Min/+}* 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 from 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 preclinical 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 *APC^{Min/+}* mice 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 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 from the present study suggest 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 *O*⁶-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 short-term study, the inhibition of ACF during 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 an advantage for its use in chemoprevention.

Acknowledgements

This work was supported by the National Health and Medical Research Council (NHMRC) and Cancer Council of South Australia

(CCSA) (project no: 1007501 and 525925). The authors thank Dr. W. Wechter for providing flurbiprofen and Olga Penino for her assistance in animal experiments.

References

- 1 Wiseman M: The second World Cancer Research Fund/ American Institute for Cancer Research expert report. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. *Proc Nutr Soc* 67: 253-256, 2008.
- 2 Reddy BS, Kawamori T, Lubet RA, Steele VE, Kelloff GJ and Rao CV: Chemopreventive efficacy of sulindac sulfone against colon cancer depends on time of administration during carcinogenic process. *Cancer Res* 59: 3387-3391, 1999.
- 3 Cuzick J, Otto F, Baron JA, Brown PH, Burn J, Greenwald P, Jankowski J, La Vecchia C, Meyskens F, Senn HJ and Thun M: Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement. *Lancet Oncol* 10: 501-507, 2009.
- 4 Murff HJ, Shrubsole MJ, Chen Z, Smalley WE, Chen H, Shyr Y, Ness RM and Zheng W: Nonsteroidal anti-inflammatory drug use and risk of adenomatous and hyperplastic polyps. *Cancer Prev Res (Phila)* 4: 1799-1807, 2011.
- 5 Moreira L and Castells A: Cyclooxygenase as a target for colorectal cancer chemoprevention. *Curr Drug Targets* 12: 1888-1894, 2011.
- 6 Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SI and Rigas B: Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol* 52: 237-245, 1996.
- 7 Piazza GA, Keeton AB, Tinsley HN, Gary BD, Whitt JD, Mathew B, Thaiparambil J, Coward L, Gorman G, Li Y, Sani B, Hobrath JV, Maxuitenko YY and Reynolds RC: A novel sulindac derivative that does not inhibit cyclooxygenases but potently inhibits colon tumor cell growth and induces apoptosis with antitumor activity. *Cancer Prev Res (Phila)* 2: 572-580, 2009.
- 8 Kashfi K and Rigas B: Non-COX-2 targets and cancer: expanding the molecular target repertoire of chemoprevention. *Biochem Pharmacol* 70: 969-986, 2005.
- 9 Piazza GA, Rahm AK, Finn TS, Fryer BH, Li H, Stoumen AL, Pamukcu R and Ahnen DJ: Apoptosis primarily accounts for the growth-inhibitory properties of sulindac metabolites and involves a mechanism that is independent of cyclooxygenase inhibition, cell cycle arrest, and p53 induction. *Cancer Res* 57: 2452-2459, 1997.
- 10 Elder DJ, Halton DE, Hague A, and Paraskeva C: Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clin Cancer Res* 3: 1679-1683, 1997.
- 11 Tinsley HN, Grizzle WE, Abadi A, Keeton A, Zhu B, Xi Y and Piazza GA: New NSAID Targets and Derivatives for Colorectal Cancer Chemoprevention. *Recent Results Cancer Res* 191: 105-120, 2012.
- 12 Piazza GA, Alberts DS, Hixson LJ, Paranka NS, Li H, Finn T, Bogert C, Guillen JM, Brendel K, Gross PH, Sperl G, Ritchie J, Burt RW, Ellsworth L, Ahnen DJ and Pamukcu R: Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res* 57: 2909-2915, 1997.
- 13 McCracken JD., Wechter WJ, Liu Y, Chase RL, Kantoci D, Murray ED Jr, Quiggle DD and Mineyama Y: Antiproliferative effects of the enantiomers of flurbiprofen. *J Clin Pharmacol* 36: 540-545, 1996.
- 14 Wechter WJ, Murray ED Jr, Kantoci D, Quiggle DD, Leipold DD, Gibson KM and McCracken JD: Treatment and survival study in the C57BL/6J-APC(Min)/+(Min) mouse with R-flurbiprofen. *Life Sci* 66: 745-753, 2000.
- 15 Wechter WJ, Kantoci D, Murray ED Jr., Quiggle DD, Leipold DD, Gibson KM and McCracken JD: R-flurbiprofen chemoprevention and treatment of intestinal adenomas in the APC(Min)/+ mouse model: implications for prophylaxis and treatment of colon cancer. *Cancer Res* 57: 4316-4324, 1997.
- 16 Leipold DD, Kantoci D, Murray ED Jr., Quiggle DD and Wechter WJ: Bioinversion of R-flurbiprofen to S-flurbiprofen at various dose levels in rat, mouse, and monkey. *Chirality* 16: 379-387, 2004.
- 17 Myers C, Koki A, Pamukcu R, Wechter W and Padley RJ: Proapoptotic anti-inflammatory drugs. *Urology* 57: 73-76, 2001.
- 18 Brown WA, Skinner SA, Malcontenti-Wilson C, Vogliagis D and O'Brien PE: Non-steroidal anti-inflammatory drugs with activity against either cyclooxygenase 1 or cyclooxygenase 2 inhibit colorectal cancer in a DMH rodent model by inducing apoptosis and inhibiting cell proliferation. *Gut* 48: 660-666, 2001.
- 19 Mahmoud NN, Boolbol SK, Dannenberg AJ, Mestre JR; Bilinski RT, Martucci C, Newmark HL, Chadburn A and Bertagnolli MM: The sulfide metabolite of sulindac prevents tumors and restores enterocyte apoptosis in a murine model of familial adenomatous polyposis. *Carcinogenesis* 19: 87-91, 1998.
- 20 Samaha HS, Kelloff GJ, Steele V, Rao CV and Reddy BS: Modulation of apoptosis by sulindac, curcumin, phenylethyl-3-methylcaffeate, and 6-phenylhexyl isothiocyanate: apoptotic index as a biomarker in colon cancer chemoprevention and promotion. *Cancer Res* 57: 1301-1305, 1997.
- 21 Janssen A, Maier TJ, Schiffmann S, Coste O, Seegel M, Geisslinger G and Grosch S: Evidence of COX-2 independent induction of apoptosis and cell cycle block in human colon carcinoma cells after S- or R-ibuprofen treatment. *Eur J Pharmacol* 540: 24-33, 2006.
- 22 Grosch S, Tegeder I, Schilling K, Maier TJ, Niederberger E and Geisslinger G: Activation of c-Jun-N-terminal-kinase is crucial for the induction of a cell cycle arrest in human colon carcinoma cells caused by flurbiprofen enantiomers. *Faseb J* 17: 1316-1318, 2003.
- 23 Grosch S, Schilling K, Janssen A, Maier TJ, Niederberger E and Geisslinger G: Induction of apoptosis by R-flurbiprofen in human colon carcinoma cells: involvement of p53. *Biochem Pharmacol* 69: 831-839, 2005.
- 24 Nutrition AIO: Report of the American Institute of Nutrition ad hoc Committee on Standards for Nutritional Studies. *J Nutr* 107: 1340-1348, 1997.
- 25 Hu Y, Martin J, Le Leu R and Young GP: The colonic response to genotoxic carcinogens in the rat: regulation by dietary fibre. *Carcinogenesis* 23: 1131-1137, 2002.
- 26 Martin JE, Le Leu RK, Hu Y and 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: G860-864, 2010.

- 27 Takayama T, Nagashima H, Maeda M, Nojiri S, Hirayama M, Nakano Y, Takahashi Y, Sato Y, Sekikawa H, Mori M, Sonoda T, Kimura T, Kato J and Niitsu Y: Randomized double-blind trial of sulindac and etodolac to eradicate aberrant crypt foci and to prevent sporadic colorectal polyps. *Clin Cancer Res* 17: 3803-3811, 2011.
- 28 Brown WA, Skinner SA, Malcontenti-Wilson C, Misajon A, DeJong T, Vogliagis D and O'Brien PE: Non-steroidal anti-inflammatory drugs with different cyclooxygenase inhibitory profiles that prevent aberrant crypt foci formation but vary in acute gastrotoxicity in a rat model. *J Gastroenterol Hepatol* 15: 1386-1392, 2000.
- 29 Charalambous D, Farmer C and O'Brien PE: Sulindac and indomethacin inhibit formation of aberrant crypt foci in the colons of dimethyl hydrazine treated rats. *J Gastroenterol Hepatol* 11: 88-92, 1996.
- 30 Takayama T, Katsuki S, Takahashi Y, Ohi M, Nojiri S, Sakamaki S, Kato J, Kogawa K, Miyake H and Niitsu Y: Aberrant crypt foci of the colon as precursors of adenoma and cancer. *N Engl J Med* 339: 1277-1284, 1998.
- 31 Geisslinger G, Lotsch J, Menzel S, Kobal G and Brune K: Stereoselective disposition of flurbiprofen in healthy subjects following administration of the single enantiomers. *Br J Clin Pharmacol* 37: 392-394, 1994.
32. Jerussi TP, Caubet JF, McCray JE and Handley DA: Clinical endoscopic evaluation of the gastroduodenal tolerance to (R)-ketoprofen, (R)- flurbiprofen, racemic ketoprofen, and paracetamol: a randomized, single-blind, placebo-controlled trial. *J Clin Pharmacol* 38: 19S-24S, 1998.
- 33 Chiu CH, McEntee MF and Whelan J: Sulindac causes rapid regression of preexisting tumors in Min/+ mice independent of prostaglandin biosynthesis. *Cancer Res* 57: 4267-4273, 1997.
- 34 Bedi A, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM, Zehnbauer BA, Hamilton SR and Jones RJ: Inhibition of apoptosis during development of colorectal cancer. *Cancer Res* 55: 1811-1816, 1995.
- 35 Rigas B and Shiff SJ: Is inhibition of cyclooxygenase required for the chemopreventive effect of NSAIDs in colon cancer? A model reconciling the current contradiction. *Med Hypotheses* 54: 210-215, 2000.
- 36 Sun Y and Sinicropo FA: Selective inhibitors of MEK1/ERK44/42 and p38 mitogen-activated protein kinases potentiate apoptosis induction by sulindac sulfide in human colon carcinoma cells. *Mol Cancer Ther* 4: 51-59, 2005.
- 37 Shiff SJ, Koutsos MI, Qiao L and Rigas B: Nonsteroidal antiinflammatory drugs inhibit the proliferation of colon adenocarcinoma cells: effects on cell cycle and apoptosis. *Exp Cell Res* 222: 179-188, 1996.
- 38 Boolbol SK, Dannenberg AJ, Chadburn A, Martucci C, Guo XJ, Ramonetti JT, Abreu-Goris M, Newmark HL, Lipkin ML, DeCosse JJ and Bertagnolli MM: Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. *Cancer Res* 56: 2556-2560, 1996.
- 39 Reddy BS, Hirose Y, Lubet R, Steele V, Kelloff G, Paulson S, Seibert K and Rao CV: Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis. *Cancer Res* 60: 293-297, 2000.
- 40 Barnes CJ, Cameron IL, Hardman WE and Lee M: Non-steroidal anti-inflammatory drug effect on crypt cell proliferation and apoptosis during initiation of rat colon carcinogenesis. *Br J Cancer* 77: 573-580, 1998.
- 41 Hong MY, Lupton JR, Morris JS, Wang N, Carroll RJ, Davidson LA, Elder RH and Chapkin RS: Dietary fish oil reduces O6-methylguanine DNA adduct levels in rat colon in part by increasing apoptosis during tumor initiation. *Cancer Epidemiol Biomarkers Prev* 9: 819-826, 2000.
42. Hong MY, Chapkin RS, Davidson LA, Turner ND, Morris JS, Carroll RJ and Lupton JR: Fish oil enhances targeted apoptosis during colon tumor initiation in part by downregulating Bcl-2. *Nutr Cancer* 46: 44-51, 2003.
- 43 Le Leu RK, Brown IL, Hu Y and Young GP: Effect of resistant starch on genotoxin-induced apoptosis, colonic epithelium, and luminal contents in rats. *Carcinogenesis* 24: 1347-1352, 2003.
- 44 Le Leu RK, Hu Y and Young GP: Effects of resistant starch and nonstarch polysaccharides on colonic luminal environment and genotoxin-induced apoptosis in the rat. *Carcinogenesis* 23: 713-719, 2002.
- 45 Hu Y, Le Leu RK and Young GP: Sulindac corrects defective apoptosis and suppresses azoxymethane-induced colonic oncogenesis in p53 knockout mice. *Int J Cancer* 116: 870-875, 2005.
46. Hu Y, Le Leu RK and Young GP: Defective acute apoptotic response to genotoxic carcinogen in small intestine of APC(Min/+) mice is restored by sulindac. *Cancer Lett* 248: 234-244, 2007.
- 47 Clarke JM, Young GP, Topping DL, Bird AR, Cobiac L, Scherer BL, Winkler JG and Lockett TJ: Butyrate delivered by butyrylated starch increases distal colonic epithelial apoptosis in carcinogen-treated rats. *Carcinogenesis* 33: 197-202, 2012.
- 48 Hu Y, McIntosh GH, Le Leu RK, Woodman R and Young GP: Suppression of colorectal oncogenesis by selenium-enriched milk proteins: apoptosis and K-ras mutations. *Cancer Res* 68: 4936-4944, 2008.

Received July 4, 2013

Revised July 16, 2013

Accepted July 17, 2013