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Advances in Brief

Evaluation of Cyclooxygenase-2 Inhibitor for Potential Chemopreventive Properties in Colon Carcinogenesis[1]

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Abstract

Epidemiological and laboratory studies indicate an inverse relationship between the risk of colon cancer development and intake of nonsteroidal antiinflammatory agents, including aspirin. One of the mechanisms by which nonsteroidal antiinflammatory agents inhibit colon carcinogenesis is through the inhibition of prostaglandin production by cyclooxygenase isozymes (COX-1 and COX-2). Overexpression of COX-2 has been observed in colon tumors. Thus, selective inhibitors of COX-2 could potentially serve as chemopreventive agents. We have assessed the chemopreventive properties of SC-58635, a COX-2 inhibitor, and of sulindac, as a positive control, in a double-blind study, using azoxymethane-induced colonic aberrant crypt foci (ACF) as a measure of efficacy. Five-week-old male F344 rats were fed the control diet (modified AIN-76A) or experimental diets containing 150 or 1500 ppm SC-58635, 320 ppm sulindac, or 1500 ppm placebo. Two weeks later, all animals except those in vehicle (normal saline)-treated groups were s.c. injected with azoxymethane (15 mg/kg of body weight, once weekly for 2 weeks). At 16 weeks of age, all rats were sacrificed and colons were evaluated for ACF. As expected, dietary administration of sulindac suppressed ACF development as such and reduced crypt multiplicity in terms of number of aberrant crypts/focus. Administration of 1500 ppm SC-58635 inhibited total ACF induction and crypt multiplicity by about 40–49%. Our finding that SC-58635 significantly suppressed colonic ACF formation and crypt multiplicity strengthens the hypothesis that a selective COX-2 inhibitor possesses chemopreventive activity against colon carcinogenesis.

Introduction

Large bowel cancer is one of the leading causes of cancer deaths in both men and women in Western countries, including the United States (1). Although several epidemiological and experimental studies suggest a relationship between the risk of development of colon cancer and dietary factors (2), recent epidemiological investigations also indicate an inverse relationship between the intake of NSAIDs,[3] specifically aspirin, and colorectal cancer risk (3, 4). Studies in laboratory animal models have also demonstrated colon tumor inhibition by several NSAIDs, including aspirin, piroxicam, sulindac, sulindac sulfone, and ibuprofen, to cite a few (5–8). Clinical studies in patients with familial adenomatous polyposis indicate that administration of sulindac causes a reduction of polyps (9).

The mechanism(s) by which NSAIDs inhibit colon carcinogenesis is not clearly understood but could possibly involve blockage of COXs, which, in turn, suppresses eicosanoid production, and especially the type 2 series of eicosanoids that affect cell proliferation, tumor growth, and immune responsiveness (10–12). In addition to inhibiting PG production, sulindac significantly blocks the formation of lipoxygenase metabolites such as 8(S)- and 12(S)-hydroxyeicosatetraenoic acid in the colonic mucosa. Sulindac also inhibits tumor developments in F344 rats and increases apoptosis in Min mice (5, 7). Recent studies have clarified that sulindac sulfone, a metabolite of sulindac, does not inhibit COX but induces apoptosis (13). Thus, it is possible that the changes in the activities of the COX and/or lipoxygenase pathways of arachidonic acid metabolism and/or modulation of events other than COX inhibition by NSAIDs may alter tumorigenesis.

At least two COX isozymes, namely COX-1 and COX-2, have been identified in colonic tumors of humans and rats (14–16). COX-1 isozyme is believed to be a constitutively expressed gene in most tissues to generate PGs for normal physiological functions; thus, the expression of this isozyme does not fluctuate due to stimuli, whereas the expression of COX-2 can be induced by a variety of agents, including growth factors and tumor promoters (15, 17, 18). Tsujii and DuBois (19) have shown that intestinal epithelial cells overexpressing the *COX-2* gene develop altered adhesion properties and resist undergoing apoptosis; these changes are reversed by treatment with NSAIDs, indicating that overexpression of the isozyme may alter the potential for development of neoplasm of intestinal epithelial cells. Also, there is evidence that the sensitivity of recombinant COX-2 toward inhibition by NSAIDs is different from that of COX-1 (20, 21). These observations raise the possibility that selective inhibitors of COX-2 could potentially serve as chemopreventive agents in colorectal carcinogenesis.

ACF, which are recognized as early preneoplastic lesions, have consistently been observed in experimentally induced colon carcinogenesis in laboratory animals (22–25). Pretlow *et al.* (26) have also shown that these lesions are present in the colonic mucosa of patients with colon cancer and have suggested that aberrant crypts are putative precursor lesions from which adenomas and carcinomas may develop in the colon (23). ACF express mutations in the *apc* gene and *ras* oncogene that appear to be biomarkers of colon cancer development (24, 27). There is some evidence that several inhibitors of ACF formation reduce the incidence of colon tumors in laboratory animals (24–25), suggesting that ACF induction can be used to evaluate novel agents for their potential chemopreventive properties against colon cancer.

The present double-blind study was designed to evaluate the inhibitory activity of SC-58635, a COX-2 inhibitor, on AOM-induced ACF formation in the colon of male F344 rats. The major goal of this study was to determine whether this compound is conceivably an effective chemopreventive agent in preclinical efficacy studies and, eventually, in human clinical trials.

Materials and Methods

Selectivity Assay and Dose Selection. Before evaluating SC-58635 for its potential chemopreventive activity, an *in vitro* assay was performed to determine the selective inhibition of COX-1 and COX-2 activities by this agent. For

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[3] The abbreviations used are: NSAID, nonsteroidal antiinflammatory drug; COX, cyclooxygenase; ACF, aberrant crypt foci; AOM, azoxymethane; PG, prostaglandin.

Table 1 *Percentage composition of experimental semipurified diets*

Ingredients	% composition	
	Control diet[a]	Experimental diet[b]
Casein	20.0	20.0
DL-Methionine	0.3	0.3
Corn starch	52.0	52.0
Dextrose	13.0	13.0
Corn oil	5.0	5.0
Alphacel	5.0	5.0
Mineral Mix, AIN	3.5	3.5
Vitamin Mix, AIN revised	1.0	1.0
Choline bitartrate	0.2	0.2
Test compounds	0	SC-58635, 150 or 1500 ppm Sulindace, 320 ppm Placebo, 1500 ppm

[a] Adapted from American Institute of Nutrition Reference Diet (AIN-76A) with the modification of source of carbohydrate.
[b] Test compounds were added to the diets at the expense of cornstarch.

the selectivity assay, insect cells expressing either COX-1 or COX-2 were homogenized and incubated with arachidonic acid (10 μM). The activities of COX-1 and COX-2 were then determined by monitoring prostaglandin E_2 (PGE_2) production (28). To measure the selective inhibition of COX-1 or COX-2 by SC-58635, 0.001–100 μM levels of this agent were preincubated with crude 1% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate homogenates (2–10 μg protein) for 10 min prior to the addition of arachidonic acid. The PGE_2 formed was detected by ELISA after a 10-min incubation period (28). The formation of PGE_2 in insect cells expressing COX-1 and COX-2 upon treatment with SC-58635 was 13 ± 3.0 and 0.04 ± 0.01 μM (mean ± SD; n = 5), respectively, indicating that SC-58635 selectively inhibited COX-2 activity.

For dose selection, the adjuvant-induced arthritis-chronic inflammatory model was used to establish the therapeutic blood level of SC-58635. Adjuvant arthritis was induced in male Lewis rats (body weight, 150–175 g, Harlan Sprague-Dawley, Inc.) by injecting 1 mg of *Mycobacterium butyricum* (Difco) in 50 μl of mineral oil into the right hind footpad. The left contralateral footpad volume was measured by the water displacement method 14 days after injection. Animals with paw volumes that were 0.4 ml greater than normal were then randomized and treated with SC-58635, beginning 15 days after adjuvant injection. The drug administration was continued until day 25 postadjuvant injection, and the mean inhibition values on paw volume were determined on the basis of eight Lewis rats. Plasma samples were collected on the final day of dosing and were analyzed for SC-58635; the therapeutic blood level was established as the lowest dose of the drug that produced the maximal antiinflammatory effect (29). SC-58635 produced a maximal effect in rats in this chronic model at a dose of 0.9 mg/kg body weight, which corresponded to a plasma level of approximately 0.3 μg/ml. SC-58635 has a markedly improved safety profile in the rat, presumably as a function of its selectivity for inducing the COX-2 enzyme while sparing the physiologically important COX-1 in the gastrointestinal tract. Therefore, studies were designed to evaluate the effect of SC-58635 on blocking ACF formation and progression at two dose levels, namely 150 and 1500 ppm, when added to the diet. These doses produced plasma levels of SC-58635 of approximately 0.5 and 3.5 μg/ml, respectively.

Animals, Diets, Carcinogen and Chemopreventive Agents. AOM (CAS:25843-45-2) was purchased from Ash Stevens (Detroit, MI). SC-58635 and placebo were coded and supplied by G. D. Searle Research and Development, (St. Louis, MO). Sulindac, a known inhibitor of colon carcinogenesis, was included in the current study as a positive control (7). Weanling male F344 rats were purchased from Charles River Breeding Laboratories (Kingston, NY). All ingredients of the semipurified diet were obtained from Dyets Inc., (Bethlehem, PA) and were stored at 4°C until the experimental diets were prepared. The rats were held in quarantine for 1 week and had access to modified AIN-76A semipurified control diet (Table 1). They were randomly distributed by weight into various dietary groups and were transferred to an animal holding room where they were housed in plastic cages, three rats/cage, under controlled conditions of a 12 h light/12 h dark cycle, 50% relative humidity, and 21°C room temperature. Experimental diets were prepared by mixing SC-58635, sulindac, and placebo with modified AIN-76A control diet.

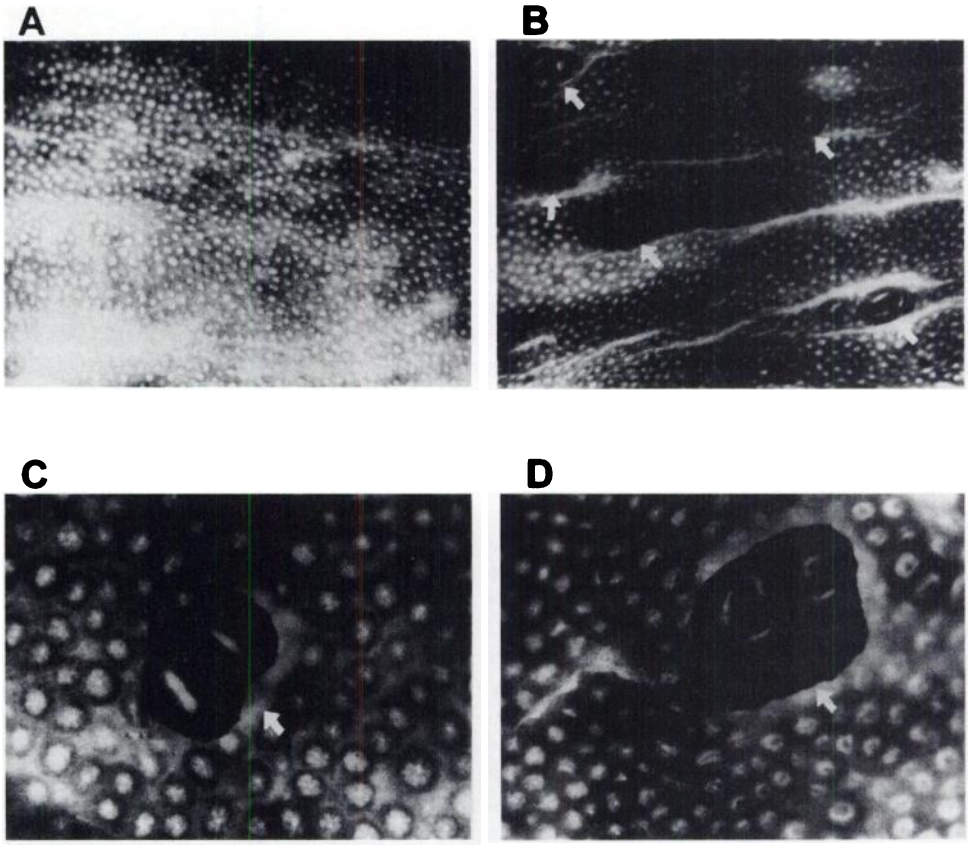

Fig. 1. Unsectioned methylene blue-stained rat colon. *A*, topographic view of colon mucosa of saline-treated control animal. Note that no ACF are identified. ×40. *B*, several AOM-induced ACF with two or more aberrant crypts/focus are identified (*arrows*). ×40. *C*, two aberrant crypts/focus. ×100. *D*, multicrypts/focus. ×100.

Table 2 *Effect of SC-58635 and sulindac on AOM-induced ACF formation in male F344 rats*[a]

Experimental group	Total no. of ACF/rat	No. of foci containing			
		1 crypt	2 crypts	3 crypts	4 or more crypts
AOM-treated[b]					
Control diet	120 ± 15	16 ± 6.5	35 ± 7.7	34 ± 4.6	35 ± 7.9
SC-58635, 1500 ppm	71 ± 15[c]	10 ± 4.5[d]	22 ± 6.8[c]	20 ± 6.8[c]	18 ± 5.8[c]
SC-58635, 150 ppm	127 ± 13	16 ± 4.6	44 ± 7.0	35 ± 6.8	33 ± 6.6
Sulindac, 320 ppm	77 ± 14[c]	11 ± 6.3[d]	24 ± 8.5[c]	21 ± 6.6[c]	21 ± 5.8[c]
Placebo, 1500 ppm	111 ± 35	15 ± 7.7	34 ± 11.8	31 ± 10.1	31 ± 10.2
Saline-treated[b]	0	0	0	0	0

[a] Values are means ± SD (n = 12).
[b] Animals were fed either control diets or experimental diets containing SC-58635, sulindac, or placebo and treated with AOM or saline (vehicle).
[c] Significantly different from control diet group treated with AOM; $P < 0.001$.
[d] Significantly different from control diet group treated with AOM; $P < 0.05$.

SC-58635 and the nature of the placebo were not identified until completion of the study.

Experimental Procedure. At 5 weeks of age, groups of animals were fed the modified AIN-76A (control) or experimental diets containing 150 or 1500 ppm SC-58635, 1500 ppm placebo, or 320 ppm sulindac. At 7 weeks of age, all animals except the vehicle-treated rats received AOM s.c. once weekly for 2 weeks at a dose rate of 15 mg/kg body weight per week. Animals intended for vehicle treatment were given an equal volume of normal saline. The rats were continued on control and experimental diets until the termination of the study, when they were 16 weeks of age. All animals were sacrificed by CO_2 euthanasia. The colons were removed, flushed with Krebs-Ringer solution, opened from cecum to anus, and fixed flat between two pieces of filter paper in 10% buffered formalin. Microscope slides were placed on the top of the filter paper to ensure that the tissue remained flat during fixation. After a minimum of 24 h in buffered formalin, the colons were cut into 2-cm segments, starting at the anus; for the next 5–10 min they were placed in a Petri dish containing 0.2% methylene blue in Krebs-Ringer solution. They were then placed, mucosal side up, on a microscope slide and observed through a light microscope. ACF were recorded according to standard procedures that are being used routinely in our laboratory (22–25). Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from lamina to basal surface of cells, and the easily discernible pericryptal zone (Fig. 1). The parameters used to assess the aberrant crypts were occurrence and multiplicity. Crypt multiplicity was determined as the number of crypts in each focus and categorized as those containing up to three, or four or more aberrant crypts/focus. All colons were scored by one observer without knowing the identity of agents under study; scores were checked at random by a second observer.

Statistical Analysis. All results were expressed as the means ± SD and were analyzed by one-tailed Student's t-test. Differences were considered statistically significant at $P < 0.05$.

Results

General Observation. The test agent and placebo were decoded only after completion of ACF scoring. The body weights of rats treated with vehicle or AOM and fed the control and experimental diets were comparable throughout the study period (data not shown). In vehicle-treated animals, administration of experimental diets containing SC-58635, sulindac, or placebo did not produce any gross changes in the liver, kidney, intestine, and lungs.

Aberrant Crypts. Those animals receiving saline and being fed the control or experimental diets showed no evidence of ACF formation in the colon (data not shown). In rats fed the control diet, AOM treatment induced, on the average, about 120 ACF/colon and 35 foci containing multiple (4 or more) aberrant crypts/focus (Table 2). ACF were predominantly observed in the distal colons. Efficacy end points used in this study were inhibition of total occurrences of ACF and reduction of number of multicrypt clusters (four or more) of aberrant crypts. In the present study, sulindac, which has been shown to be a strong inhibitor of colon carcinogenesis in animal assays and has reduced polyps in patients with familial polyposis, was also found to be an effective inhibitor of total occurrences of ACF/colon (36%) and of multicrypt clusters containing two, three, or even four or more crypts/focus (31–40%). Administration of 1500 ppm SC-58635 significantly suppressed the total number of ACF/colon (40% inhibition) as compared to control diet. Aberrant crypt multiplicities per focus were significantly decreased (41–49%). The lower dose of SC-58635 had only minimal impact on ACF formation. As expected, administration of placebo had no effect on ACF inhibition.

Discussion

The prolonged administration of NSAIDs has been associated with side effects such as gastrointestinal ulceration and bleeding, as well as renal toxicity. Because aspirin, sulindac, piroxicam and indomethacin, commonly used NSAIDs, have little or no selectivity for inhibition of COX-1 or COX-2 activity (30, 31), the development of more specific and potent, yet minimally toxic, inhibitors of COX-2 may provide useful cancer chemopreventive agents. The present study was undertaken to evaluate the selective COX-2 inhibitor SC-58635 for its potential chemopreventive activity against ACF formation in the colon. ACF are putative preneoplastic lesions. Because multiplicity of four or more aberrant crypts/focus has been a consistent predictor of colon tumor outcome (32, 33), the present study used this criterion to evaluate SC-59635 for its potential chemopreventive properties. The results of this study support our earlier efficacy study with sulindac (7) and provide additional evidence that crypt multiplicity and ACF are predicative of colon tumor incidence. Sulindac was found to be a strong inhibitor of chemically induced colon carcinogenesis in animal models and is currently under evaluation in human clinical chemoprevention trials (7, 9). COX-2 inhibitors reduce inflammatory PG synthesis without affecting PG levels in normal tissues that result from COX-1 activity (20). The fact that a selective COX-2 inhibitor like SC-58635 can inhibit colonic ACF formation and crypt multiplicity further suggests a role for similar compounds as potential chemopreventive agents against colon cancer. This finding is significant because SC-58635, through its ability to inhibit COX-2 expression, has the potential to block the development and/or progression of colon carcinogenesis, and at the same time, this agent possesses minimal or no gastric toxicity. The lack of an inhibitory effect of the low dose of SC-58635 that was effective in modulating adjuvant-induced arthritis in rats may indicate that higher blood levels of this agent are needed to achieve adequate colonic exposure; however, it is noteworthy that the higher dose of SC-58635 did not induce any symptoms attributable to toxicity. It is possible that, in addition to COX-2 inhibition, SC-58635 may also increase apoptosis, thereby inhibiting ACF formation. Further experiments, including preclinical efficacy studies, are warranted to fully evaluate this compound for its chemopreventive properties.

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