Chemopreventive Properties of a Selective Inducible Nitric Oxide Synthase Inhibitor in Colon Carcinogenesis, Administered Alone or in Combination with Celecoxib, a Selective Cyclooxygenase-2 Inhibitor

Chinthalapally V. Rao, Cooma Indranie, Barbara Simi, Pamela T. Manning, Jane R. Connor, and Bandaru S. Reddy

ABSTRACT

The inducible isoforms of nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) are overexpressed in colonic tumors of humans, as well as in colon tumors that develop in rats after the administration of the colon-specific carcinogen, azoxymethane (AOM). iNOS may regulate COX-2 production of proinflammatory prostaglandins, which are known to play a key role in colon tumor development. Experiments were designed to assess the potential chemopreventive properties of highly selective iNOS inhibitors, administered individually and in combination with a selective COX-2 inhibitor, on the development of AOM-induced colonic aberrant crypt foci (ACF). F344 rats were fed experimental diets containing one of the following: 0, 10, 30, or 100 parts/million (ppm) of the selective INOS inhibitor L-N(ω)-arginine (L-N6-arginine; SC-51); 1800 ppm of the less potent, selective INOS inhibitor aminoguanidine (AG); 500 ppm of the COX-2 inhibitor celecoxib; 320 ppm of the nonsteroidal anti-inflammatory salidroside (positive control); or 30 ppm of SC-51 with 500 ppm of celecoxib, and 100 ppm of SC-51 with 500 ppm of celecoxib. One and 2 weeks later, rats received s.c. injections of AOM at a dose of 15 mg/kg of body weight. At 17 weeks of age, all rats were sacrificed. Colonos were evaluated for ACF, and colonic mucosae were assayed for COX and NOS isoform enzyme activities. Samples of venous blood, collected at various time points, were analyzed for these agents. SC-51, administered alone, demonstrated dose-dependent inhibition of the incidence of colonic ACF. The highest doses of SC-51 (100 ppm) and AG (1800 ppm) significantly suppressed the incidence of colonic ACF (P < 0.01 and < 0.001, respectively) and crypt multiplicity in terms of numbers of aberrant crypts/focus (P < 0.0001). Importantly, the combination of either low or high effective doses of SC-51 (30 or 100 ppm) and celecoxib (500 ppm) suppressed AOM-induced colonic ACF formation (P < 0.05 and < 0.001, respectively) and reduced multiplicity of four or more aberrant crypts/focus (P < 0.0001) to a greater extent than did these agents administered individually. As expected, sulindac inhibited colonic ACF formation (P < 0.01) and reduced the multiplicity of four or more aberrant crypts (P < 0.0001) to −45%. The enzymatic activities of COX-2 and iNOS were significantly induced in the AOM-treated animals, and administration of both the iNOS inhibitors, SC-51 and AG, significantly inhibited the activities of both iNOS and COX-2 in the colonic mucosa. The combined administration of SC-51 and celecoxib inhibited the COX-2 activity to a greater extent than did either of these agents administered alone. These findings support the hypothesis that selective iNOS inhibitors may have chemopreventive properties and that coadministration with a selective COX-2 inhibitor may have additional chemopreventive potential.

INTRODUCTION

Colorectal cancer is one of the leading causes of cancer deaths in both men and women in Western countries, including the United States (1). Epidemiological and experimental studies indicate that the risk of developing colon cancer may be attributable to combined actions of environmental factors and endogenous promoting agents (2). Recently, much attention has been given to endogenous factors, which appear to be responsible for tumor cell growth, spreading, and invasion (progression and metastasis). Identifying such endogenous factors should lead not only to a better understanding of the processes of tumor cell progression and metastasis, but may also provide new strategies for developing agents that specifically suppress these processes.

The protective role of NSAIDs (3) such as aspirin, piroxicam, and sulindac in colon cancer has been well documented in epidemiological and animal studies. Collectively, these studies demonstrate an inverse relationship between the use of NSAIDs and colon cancer (3). Accumulating evidence indicates that the inhibition of colon tumor development by NSAIDs is mediated through the modulation of AA metabolism via COX enzymes, which in turn inhibits immune responsiveness (4, 5). Prolonged administration of NSAIDs, however, can cause gastrointestinal bleeding and ulceration as well as renal toxicity, resulting primarily from the inhibition of constitutive COX-1 activity (6). NSAIDs inhibit the activities of both COX-1 and -2, accounting for their chemopreventive activity as well as the adverse side effects. As a result, there was a rationale to develop selective COX-2 inhibitors that block inducible COX-2 activity but that spare COX-1 activity and the normal physiological functions of this enzyme (7, 8). We have previously demonstrated that a COX-2 selective inhibitor, celecoxib, suppressed induction of the colonic ACF by AOM and inhibited colon tumor formation (9, 10). We have also shown that COX-2 inhibitors such as nimesulide and nabumetone inhibited the development of colonic ACF (11). Additional evidence in support of an inhibitory role of COX-2 has been demonstrated in studies showing that MF-Tricyclic, a COX-2 inhibitor, blocked intestinal tumorigenesis in APCΔ716 mice (12). Recently, celecoxib has been approved for the treatment of patients with familial adenomatous polyposis who carry a mutation similar to that in the APC mouse (13, 14). Extensive studies conducted over the past few years on COX-2 have greatly improved our understanding of its role in colorectal cancer and other diseases (15). Mechanistic studies in our laboratory and elsewhere support the hypothesis that COX-2 regulation is highly complex and influenced by various exogenous and endogenous factors, including NO (7–15).

NO is produced endogenously during arginine metabolism by different isoforms of NOS, enzymes possessing a wide range of physiological and pathophysiological actions (16). iNOS, the distinct inducible, Ca2+-independent isoform of NOS (130-kDa protein) can be expressed in response to proinflammatory agents. This isoform produces high and sustained concentrations of NO, compared with the

Received 8/1/01; accepted 11/1/01.

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1 Supported in part by Pharmacia Corp. (St. Louis, MO).
2 To whom requests for reprints should be addressed, at the American Health Foundation, One Dana Road, Valhalla, NY 10595. E-mail: crao@ahf.org.

3 The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; AA, arachidonic acid; COX, cyclooxygenase; ACF, aberrant crypt foci; AOM, azoxymethane; iNOS, inducible nitric oxide synthase; ppm, parts/million; PG, prostaglandin; AG, aminoguanidine.
low, transient levels of NO produced by the Ca\(^{2+}\)-dependent neuronal and endothelial isoforms (17, 18). Studies from our group and others suggest that iNOS may play a role in tumor development (19). Increased NOS expression and/or activity were reported in human gynecological (20), breast (21), and central nervous system (22) tumors. Furthermore, iNOS expression and nitrotyrosine accumulation (a marker of peroxynitrite, the product of NO and superoxide) in inflamed mucosa of patients with ulcerative colitis and gastritis demonstrate the production of NO and its potential involvement in the pathogenesis of these diseases (23). In addition, Amb's et al. (24) showed increased expression and activity of iNOS in human colon adenomas. Studies in experimental models of colon cancer indicate that AOM-induced colon tumors have higher expression and/or activity of iNOS compared with levels found in adjacent colonic tissue (25, 26). The role of NO in cancer is not well defined and appears to be complex. NO can produce damage to DNA, either directly or indirectly by several mechanisms (27), interfere with DNA repair (28), and/or cause post-translational modification, potentially leading to tumor initiation and promotion. It is therefore probable that the high, sustained levels of NO generated by iNOS can produce multiple types of damage and, in chronic conditions, lead to an accumulation of gene mutations, including mutation of the tumor suppressor gene p53 (29), and alterations in cellular function. Once the tumor is established and progresses, the data suggest that NO may also mediate protumorigenic activities, including capillary leakiness, angiogenesis, and leukocyte adhesion and infiltration, and as a result, eventually metastasis (30). Importantly, iNOS has been shown to be involved in the regulation of COX-2 activity, which plays a pivotal role in colon tumorigenesis (31). Taken together, these observations suggest that iNOS may play a critical role in colon tumorigenesis. In support of this hypothesis, we have shown previously that the iNOS-selective inhibitor, S\(S'S',1.4\)-phenylene-bis(1.2-ethanediyl)bis-isothiourea, prevented AOM-induced colon ACF development and iNOS activity in this model (19).

ACF, which are recognized as early preneoplastic lesions, develop in experimentally induced colon carcinogenesis in laboratory animals (32). Pretlow et al. (33) have also shown the presence of such lesions in the colonic mucosa of patients with colon cancer and have suggested that aberrant crypts are precursor lesions from which adenomas and carcinomas develop in the colon. ACF express mutations in the Apc gene and ras oncogene, which appear to be biomarkers of colon cancer development (34). Importantly, our preliminary studies indicate that COX-2 and iNOS were overexpressed in the AOM-induced colon ACF (11, 25). There is also evidence that several inhibitors of ACF development reduce colon tumorigenesis in laboratory animals (35, 36).

There is increasing interest in the application of combinations of low doses of chemopreventive agents with different modes of action as a means of obtaining increasing efficacy while minimizing toxicity. Previously, we documented that lower doses of piroxicam, an NSAID, and difluoromethylornithine, an ornithine decarboxylase inhibitor, administered together were more effective in inhibiting colon tumorogenesis than when these agents were given individually at higher doses (37).

The present double-blind study was designed to evaluate the potential chemopreventive efficacy of a selective iNOS inhibitor, L-\(N'\)-(1-iminoethyl)lysine tetrazole-amide (SC-51), alone and in combination with celecoxib against AOM-induced ACF formation. In addition, to contribute to our understanding of the mechanism of inhibition of these compounds, the modulation of COX and NOS enzyme activities by these agents was assessed.

## MATERIALS AND METHODS

### Animals, Diets, Carcinogen, and Chemopreventive Agents

AOM (CAS no. 25843-45-2) was purchased from Ash Stevens (Detroit, MI). iNOS and COX-2 selective inhibitors were provided by Pharmacia Corporation, (St. Louis, MO). Sulindac was supplied by the Chemoprevention Branch of the National Cancer Institute (Bethesda, MD). 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 prior to the initiation of the study and had access to modified AIN-76A semipurified control diet (37). They were randomly distributed into various dietary groups and were transferred to an animal holding room where they were housed, three rats to a plastic 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 chemopreventive agents with modified AIN-76A control diet.

### Experimental Procedure

At 5 weeks of age, groups of male F344 rats (18–23 rats/group) were fed the modified AIN-76A (control) or experimental diets containing 10, 30, or 100 ppm SC-51; 1800 ppm AG; or 500 ppm celecoxib. Other groups of the same size were given 30 ppm SC-51 + 500 ppm celecoxib, 100 ppm SC-51 + 500 ppm celecoxib, or 320 ppm (80% maximum tolerated dose) sulindac (positive control). At 7 weeks of age, all animals except the vehicle-treated control rats received AOM by s.c. injection once weekly for 2 weeks at a dose of 15 mg/kg of body weight per week. Control animals intended for vehicle treatment were given an equal volume of normal saline. One day prior to the first AOM injection and 4 and 8 weeks after the second AOM treatment, while the rats were maintained on control or experimental diets, blood samples were taken from animals in each group from the ocular vein under halothane anesthesia for analysis of iNOS and COX-2 inhibitors. All rats were killed by CO\(_2\) euthanasia 8 weeks after the second AOM injection. The colons were removed (12/group), flushed with Krebs Ringer solution, opened from cecum to anus, and fixed flat between two pieces of filter paper in 10% buffered formalin for ACF analysis. For the analysis of Ca\(^{2+}\)-dependent and -independent NOS as well as COX-1 and COX-2 activities, rats were killed by CO\(_2\) euthanasia, and their colonic mucosae were scraped, frozen under liquid N\(_2\), and stored at −70°C for further analysis.

### ACF Analysis

After a minimum of 24 h in buffered formalin, the colons were cut into 2-cm segments, starting at the anus, and placed in a Petri dish containing 0.2% methylene blue in Krebs Ringer solution for 5–10 min. They were then placed, mucosal side up, on a microscope slide and observed through a light microscope. ACF were recorded according to standard procedures used routinely in our laboratory (19, 36). Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from lamina to basal surfaces of cells, and the easily discernible pericryptal zone. The parameters used to assess the aberrant crypts were their occurrence and multiplicity. Crypt multiplicity was determined as the number of crypts in each focus and categorized as containing up to three or four more aberrant crypts/focus. All colons were scored by one observer who did not know the identity of agents under study. Scores were checked at random by a second observer.

### Assay of Calcium-dependent (Constitutive NOS) and -independent (iNOS) Activity

Constitutive NOS and iNOS activities were determined in colonic mucosal samples. Conversion of \(L\)-arginine to \(L\)-citrulline was measured by a modification described previously (19, 24). The assay was carried out by adding 100 \(\mu\)g of sample protein to 150 \(\mu\)l of assay buffer [50 mM HEPES, 1 mM DTT, 1 mM MgCl\(_2\), 5 mM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 3 mM aprotinin (pH 7.4)] containing 70 \(\mu\)M arginine, 250,000 dpm \(\lceil6^\text{H}\rceil\)arginine, 2 mM NADPH, 5 mM tetrahydrobiopterin, 5 \(\mu\)M flavin adenine dinucleotide, and 0.5 mM CaCl\(_2\) to measure total NOS activity, or in the presence of 1 mM EGTA (without calcium) to determine Ca\(^{2+}\)-independent iNOS activity. After 30 min at 37°C, the reaction was stopped with 100 \(\mu\)l of 1 \(N\) trichloroacetic acid. The samples were adjusted to pH 4.6 by adding 500 \(\mu\)l of 20 mM HEPES and applied to Dowex AG 50W-X8 resin columns. \(\lceil6^\text{H}\rceil\)Citrulline was eluted and separated by TLC. Radioactivity was counted with a BioScan Radiomatic detector. Results are expressed as pmol \(\lceil6^\text{H}\rceil\)citrulline/mg protein/min.
COX-1 and COX-2 Activity. Colonic mucosae from individual rats were homogenized in 1:3 (w/v) volumes of 100 mM Tris-HCl buffer (pH 7.2), using a Polytron tissue homogenizer. The samples were then centrifuged at 9000 × g at 4°C for 10 min, and the supernatant fraction was centrifuged at 100,000 × g for 1 h. The resulting microsomal pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.4) for COX activity assays. COX activities in microsomal fractions were assayed using a slight modification of previously published methods (19, 35). Briefly, 150 μl of the reaction mixture contained 12 μM [14C]-AA (44,000,000 dpm), 1 mM epinephrine, 1 mM glutathione in 50 mM phosphate buffer (pH 7.4), and 20–30 μg of microsomal protein. For measuring COX-1 activity, proteins were preincubated with 25 μM celecoxib, a COX-2 specific inhibitor, to block the COX-2 isoform activity. For determining COX-2 activity, proteins were preincubated with 50 μM aspirin to block the activity of COX-1. After incubation at 37°C for 15 min, the reaction was terminated by adding 40 μl of 0.25 M HCl. The COX-mediated metabolites of AA were extracted three times with ethyl acetate (0.5 ml each time). The combined extracts were evaporated to dryness under N₂, redissolved in chloroform, and subjected to TLC on precoated plastic TLC plates (silica G60; 125 × 10 × 0.25 mm). The plates were developed with a solvent system containing chloroform-methanol-acetic acid (100:15:1; v/v/v) and exposed in an iodide chamber for 5 min to visualize the standards. The metabolites of 14C- AA corresponding to PGE₂, PGF₂α, PGF₃, 6-keto-PGF₁α, and thromboxane B₂ were detected by their comigration (Rf values) with authentic standards. The area of each metabolite was determined in a Bioscan System 2000 image scanning counter (Bioscan Inc., Washington, DC) equipped with a beta detector.

Analysis of Plasma for Celecoxib and SC-51. To correlate the dose with the pharmacological effects of celecoxib and SC-51 in rats fed either or both agents, plasma levels were examined at various time points as indicated above. Celecoxib was measured in plasma as described previously, using reverse-phase high-performance liquid chromatography and a fluorescence detector with excitation at 240 nm and emission at 380 nm (10). To assess SC-51, plasma samples (0.2 ml) containing an internal standard were acidified with 1:10 ammonium hydroxide-methanol (v/v), and the eluates were evaporated to dryness under N₂, redissolved in 20 × 20 mM; layer thickness, 150 μm). The TLC plates were developed with a solvent system containing chloroform-methanol-acetic acid-water (100:15:1.25:1, v/v/v/v) and exposed in an iodide chamber for 5 min to visualize the standards. The metabolites of 14C- AA corresponding to PGE₂, PGF₂α, PGD₂, 6-keto-PGF₁α, and thromboxane B₂ were detected by their comigration (Rf values) with authentic standards. The area of each metabolite was determined in a Bioscan System 2000 image scanning counter (Bioscan Inc., Washington, DC) equipped with a beta detector.

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

RESULTS

The body weights of rats treated with vehicle or AOM and fed the control or experimental diets containing the test agents were comparable throughout the study period (data not shown). In vehicle-treated rats, the experimental diets containing the selective iNOS inhibitors, COX-2 inhibitor, their combination, or sulindac did not produce any observable toxicity or any gross changes in the liver, kidney, intestine, and lungs.

The control rats treated with saline and fed the control or experimental diets showed no evidence of ACF formation in the colon (data not shown). AOM-treated rats fed the control diet had ~120 ACF/colon and 29 foci that contained multiple (four or more) aberrant crypts/focus (Table 1). ACF were observed predominantly in the distal colons. The inhibition of ACF occurrence, as well as reduction of the number of multicrypt clusters (four or more) of aberrant crypts, was used to define efficacy end points in this study. As expected, administration of sulindac (positive control), a nonselective COX inhibitor, was an effective inhibitor of total ACF/colon (30% reduction; P < 0.001) and of multicrypt clusters containing four or more aberrant crypts/focus (43% decrease; P < 0.0001). None of the test agents had any measurable impact on the formation of one or two aberrant crypt foci formation; however, inhibitor effects were seen with respect to formation of three crypt foci (Table 1). Administration of the lower doses (10 or 30 ppm) of SC-51 had a minimal inhibitory effect on the total ACF, as did 500 ppm celecoxib (5–20% inhibition), confirming previous results in this model with this compound. However, 100 ppm SC-51 and 1800 ppm AG significantly suppressed AOM-induced total colonic ACF (P < 0.01 and 0.001, respectively) and multicrypt clusters of four or more crypts (P < 0.0001) compared with the control diet. Furthermore, the data on the colonic ACF with the selective iNOS inhibitors suggest a trend toward greater suppression of crypt multiplicities than of total ACF inhibition. As shown in Fig. 1, the number of crypt multiplicities of four or more was reduced in a dose-dependent manner by SC-51. In addition, the highest dose of SC-51, as well as AG, reduced the number of crypt multiplicities to a degree comparable to that produced by the positive control, sulindac. Importantly, the combination of the iNOS and COX-2 inhibitors at submaximal effective doses significantly decreased total colonic ACF (P < 0.001) and aberrant crypt multiplicities (four or more per focus; P < 0.0001). The combination of 30 ppm SC-51 with 500 ppm celecoxib reduced the number of foci containing four or more crypts to the maximal effect that could be obtained with the positive control, sulindac (Fig. 2).

The modulation of colonic mucosal enzyme activities of NOS and COX isoforms by iNOS and COX-2 inhibitors is summarized in Table 2. Administration of AOM significantly increased Ca²⁺-dependent NOS activity (P < 0.0001) in the colonic mucosa but had no significant effect on Ca²⁺-independent NOS activity (P > 0.05), reflecting an increase in iNOS but not in the constitutive isoform. AOM treatment significantly induced both isoforms of COX activities (P < 0.05 for COX-1 and P < 0.0001 for COX-2 activity), but the effect was more pronounced on COX-2 than on COX-1 activity (>10-fold versus 2-fold increase). SC-51 administered at 100 ppm and AG at 1800 ppm significantly suppressed the AOM-induced colonic mucosal iNOS

Table 1. Chemopreventive effect of iNOS selective inhibitors and celecoxib individually and in combination on AOM-induced colonic ACF formation

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>1 crypt</th>
<th>2 crypts</th>
<th>3 crypts</th>
<th>≥4 crypts</th>
<th>Total ACF incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.4 ± 1.9</td>
<td>30.8 ± 2.6</td>
<td>37.6 ± 2.2</td>
<td>28.9 ± 2.0</td>
<td>120 ± 6.7</td>
</tr>
<tr>
<td>10 ppm SC-51</td>
<td>28.2 ± 1.8</td>
<td>31.7 ± 2.7</td>
<td>29.3 ± 2.3⁰</td>
<td>25.6 ± 1.8</td>
<td>115 ± 4.8</td>
</tr>
<tr>
<td>30 ppm SC-51</td>
<td>28.7 ± 2.9</td>
<td>32.6 ± 1.8</td>
<td>24.8 ± 1.7⁰</td>
<td>22 ± 2.7⁰</td>
<td>108 ± 5.5</td>
</tr>
<tr>
<td>100 ppm SC-51</td>
<td>28.6 ± 2.2</td>
<td>33.6 ± 2.1</td>
<td>17.7 ± 1.1⁰</td>
<td>13.9 ± 1.1⁰</td>
<td>93.8 ± 4.2⁰</td>
</tr>
<tr>
<td>1800 ppm AG</td>
<td>28.0 ± 2.4</td>
<td>29.3 ± 1.3</td>
<td>17.8 ± 1.0⁰</td>
<td>14.6 ± 1.8⁰</td>
<td>89.8 ± 4.5⁰</td>
</tr>
<tr>
<td>500 ppm celecoxib</td>
<td>26.8 ± 2.1</td>
<td>27.3 ± 2.0⁰</td>
<td>23.7 ± 2.3⁰</td>
<td>23.3 ± 2.7⁰</td>
<td>102 ± 7.5⁰</td>
</tr>
<tr>
<td>30 ppm SC-51 + 500 ppm celecoxib</td>
<td>29.3 ± 3.0</td>
<td>31.2 ± 2.3</td>
<td>21.6 ± 2.0⁰</td>
<td>17.6 ± 1.9⁰</td>
<td>99.7 ± 7.5⁰</td>
</tr>
<tr>
<td>100 ppm SC-51 + 500 ppm celecoxib</td>
<td>26.3 ± 2.9</td>
<td>27.4 ± 2.2</td>
<td>16.3 ± 1.9⁰</td>
<td>12.6 ± 1.8⁰</td>
<td>84.3 ± 6.3⁰</td>
</tr>
<tr>
<td>320 ppm sulindac</td>
<td>23.3 ± 1.8</td>
<td>28.0 ± 1.5</td>
<td>17.3 ± 1.7</td>
<td>16.5 ± 0.9</td>
<td>84.9 ± 4.1⁰</td>
</tr>
</tbody>
</table>

* Mean ± SE (n = 12–16).

⁰ Significantly different from the control diet group by Student’s t test at: ⁰ P < 0.01; ⁰ P < 0.0001; ⁰ P < 0.001; ⁰ P < 0.05.

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activity by ~35% and 50%, respectively. The administration of 500 ppm celecoxib reduced the AOM-induced COX-2 activity by ~60%. Unexpectedly, the administration of 100 ppm SC-51 plus 500 ppm celecoxib produced an even greater reduction in COX-2 activity than did celecoxib alone (P < 0.0001).

Plasma Concentrations of Test Agents. As summarized in Table 3, the plasma levels of celecoxib administered at 500 ppm ranged from 1072 to 1706 ng/ml at various time points throughout the study. The plasma concentrations of SC-51 reflected a dose dependence (for the three time points sampled: 461, 341 and 254 ng/ml for 10 ppm; 1315, 821, and 742 ng/ml for 30 ppm; and 4306, 2811, and 2639 ng/ml for 100 ppm). Our results also demonstrate that the combined administration of 30 or 100 ppm SC-51 with 500 celecoxib did not significantly alter the exposure to either test agent.

DISCUSSION

The present study demonstrates that administration of the selective iNOS inhibitors SC-51 and AG significantly suppressed AOM-induced colonic ACF formation and colonic mucosal iNOS enzyme activity in rats. We have also shown for the first time that the coadministration of an iNOS inhibitor and a COX-2 inhibitor in the diet significantly suppresses AOM-induced colonic ACF formation. An important finding of this study is that celecoxib and SC-51, when administered together, were more effective in inhibiting ACF formation than was administration of the same doses of these agents individually. Importantly, the administration of an iNOS inhibitor plus a COX-2 inhibitor also suppressed AOM-induced colonic mucosal inducible isozymes of NOS (~25%) and COX (~45%) activities to a greater degree than when the inhibitors were given individually. The correlation of inhibition of crypt formation with the reduction in both COX-2 and iNOS enzymatic activities suggests their important role in AOM-induced tumorigenesis. It is known that expression of the inducible isozymes of NOS and COX is increased in human colorectal tumors, as well as in carcinogen-induced colon tumors in laboratory rats (24–26). The precise pathobiological functions of both iNOS and COX-2 activities in colorectal carcinogenesis are not clearly understood. Recent reports suggest that iNOS may contribute to colon tumor development or acceleration of the progression of colon carcinogenesis. Importantly, only iNOS produces sustained NO concentrations in the micromolar range, and this inducible isoform is specifically associated with neoplastic tissue. The demonstration of inhibition of early stages of colon carcinogenesis by iNOS selective inhibitors, such as AG and SC-51, further supports the hypothesis that iNOS appears to play a significant role in tumorigenesis. The results of the present study also support our previous finding with the selective iNOS inhibitor, S,S’-1,4-phenylene-bis(1,2-ethanediyl)bis-isothiourea, which we found also suppresses AOM-induced colonic ACF and activities of inducible NOS and COX (19).

The precise mechanisms involved in the suppression of AOM-induced colonic ACF by selective iNOS inhibitors are also not yet fully understood and are most likely multiple in nature, as diagramed in Fig. 3. Previous reports indicated that iNOS is associated with the modulation of COX-2 activity in colorectal cancer (23, 38, 39). NO has been found to enhance the activity of COX-2 in a variety of cell types. In addition, increased COX-2 expression and activity in colorectal tumors in humans and rodents have been reported (40, 41). Overexpression of COX-2 may increase the production of several mitogenic eicosanoids, particularly the type-2 series PGs that are involved in antiapoptotic activity in epithelial tumors (42).

Previously, we demonstrated that the COX-2 inhibitor, celecoxib, suppressed AOM-induced colonic ACF and adenocarcinomas in this model (9, 26). The precise pathobiological functions of both iNOS and COX-2 activities in colorectal carcinogenesis are not clearly understood.

### Table 2

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>NOS activity&lt;sup&gt;a&lt;/sup&gt; (pmol [H]citrulline/mg protein/min)</th>
<th>COX activity&lt;sup&gt;a&lt;/sup&gt; (pmol [14C]-AA metabolized/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-treated control</td>
<td>8.2 ± 1.4</td>
<td>≤0.1</td>
</tr>
<tr>
<td>AOM-treated control</td>
<td>10.5 ± 1.9</td>
<td>2.4 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 ppm SC-51</td>
<td>9.8 ± 1.5</td>
<td>1.6 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1800 ppm AG</td>
<td>7.9 ± 1.7</td>
<td>1.1 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>500 ppm celecoxib</td>
<td>11.3 ± 2.1</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>100 ppm SC-51 + 500 ppm celecoxib</td>
<td>7.8 ± 1.3</td>
<td>1.2 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SE (n = 4–6).

<sup>b</sup> Values in vertical columns are significantly different from vehicle control group by t test: <sup>b</sup>P < 0.0001, <sup>c</sup>P < 0.05.

<sup>c</sup> Values in vertical column are significantly different from carcinogen control group by t test: <sup>c</sup>P < 0.05; <sup>d</sup>P < 0.0001. Values in the vertical column are significantly different from 500 ppm celecoxib group by t test: <sup>d</sup>P < 0.02.
and activates the enzyme, as suggested by Marnett of NO and superoxide anion, acts as oxidant for the heme of COX-2 understood (31). It is possible that peroxynitrite, the coupling product involved in stimulation of COX-2 activity by iNOS/NO are not yet formation of proinflammatory eicosanoids. The exact mechanisms even at early stages of colon carcinogenesis. Furthermore, these results suggest that the suppression of iNOS activity by these agents may even lead to the down-regulation of COX-2 activity and decreased formation of proinflammatory eicosanoids. The exact mechanisms involved in stimulation of COX-2 activity by iNOS/NO are not yet understood (31). It is possible that peroxynitrite, the coupling product of NO and superoxide anion, acts as oxidant for the heme of COX-2 and activates the enzyme, as suggested by Marnett et al. (44). In addition to its stimulatory effects on COX-2 activity, NO or its metabolites may also affect tumor formation and/or progression by a number of other potential mechanisms. NO is an endothelial growth factor and specifically mediates the tumor vasculization to regulate blood flow (38). NO has also been found to directly or indirectly damage DNA, ultimately resulting in mutations, which may alter normal cellular processes (27). In addition, NO has been found to alter translationally modify a number of important cellular proteins, including p53, caspases, and DNA repair enzymes (28, 29). Either alone or in combination, these cellular alterations could contribute to protumorigenic phenotypes, including the formation of ACF, and ultimately to tumor formation.

The mechanism of iNOS selective inhibition by AG has been established in several studies (45, 46). In addition to iNOS modulation, AG is known to exhibit multiple NOS-independent mechanisms, including inhibition of histamine metabolism (47), polyamine metabolism (48), reduction of end product glycosylation, and inhibition of catalase activity (49). SC-51 is a more potent inhibitor of iNOS than is AG and does not exhibit additional effects on other systems. In the present study we did not evaluate the effects of AG or SC-51 on the expression of iNOS in colonic extracts. However, based on data generated in other models, it is unlikely that these agents will also influence iNOS expression (50).

The identification of the inducible isoforms of NOS and COX and their association with inflammatory and other disease states has driven efforts to develop selective inhibitors for these inducible enzymes while sparing the functions regulated by their constitutive isoforms. Because iNOS/NO and COX-2/PGs appear to be involved in the pathogenesis of colon cancer, selective inhibitors that reduce their activities may be potential chemopreventive agents. The results described here support the hypothesis that inhibition of one or both of these inducible enzymes is chemopreventive. In addition, the utilization of a novel selective iNOS inhibitor in this model supports previous data suggesting that selective iNOS inhibitors may provide safe and effective chemopreventive agents for colon cancer.

Although the administration of a combination of agents with different modes of action is not novel, this is the first study demonstrating the efficacy of coadministration of a COX-2 and an iNOS inhibitor in producing a chemopreventive effect in the colon. We have shown in several previous studies that, indeed, this combination approach with other therapeutic agents can provide greater efficacy than the individual agents administered alone (51, 52). It is anticipated that the administration of a combination of chemopreventive agents, which are selected based on definitive mechanisms relevant to tumorigenesis, should have beneficial applications in human cancer chemoprevention trials.

ACKNOWLEDGMENTS

We thank Jeff Rigotty and the technical staff of the Research Animal Facility for expert handling of efficacy studies. We thank Ilse Hoffmann for editing and Laura Nast for preparation of the manuscript.

REFERENCES


Table 3 Plasma concentrations of iNOS and COX-2 inhibitors at three time points throughout the study

<table>
<thead>
<tr>
<th>Chemopreventive agent</th>
<th>Early (1 week on diet)</th>
<th>Mid (5 weeks on diet)</th>
<th>Late (9 weeks on diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ppm SC-51</td>
<td>461 ± 15</td>
<td>341 ± 9.7</td>
<td>254 ± 13</td>
</tr>
<tr>
<td>30 ppm SC-51</td>
<td>1315 ± 71</td>
<td>821 ± 22</td>
<td>742 ± 7</td>
</tr>
<tr>
<td>100 ppm SC-51</td>
<td>4306 ± 146</td>
<td>2811 ± 82</td>
<td>2639 ± 268</td>
</tr>
<tr>
<td>500 ppm celecoxib</td>
<td>1440 ± 118</td>
<td>1706 ± 176</td>
<td>1072 ± 358</td>
</tr>
<tr>
<td>30 ppm SC-51 + 500 ppm celecoxib</td>
<td>1240 ± 37 + 1066 ± 43</td>
<td>901 ± 50 + 1660 ± 153</td>
<td>788 ± 63 + 2534 ± 96</td>
</tr>
<tr>
<td>100 ppm SC-51 + 500 ppm celecoxib</td>
<td>4370 ± 112 + 1475 ± 72</td>
<td>3096 ± 344 + 1547 ± 92</td>
<td>2477 ± 204 + 1959 ± 191</td>
</tr>
</tbody>
</table>

Values are ng/ml of plasma, mean ± SE (n = 4).

10. More recently, we have found that celecoxib suppresses AOM-induced colon cancer in a dose-dependent manner and that it halts promotion and progression of colon tumor development (43). The results of the present study demonstrate that inducible isoforms of NOS and COX are selectively inhibited in colonic mucosa by iNOS inhibitors, indicating a possible association between these enzymes even at early stages of colon carcinogenesis. Furthermore, these results suggest that the suppression of iNOS activity by these agents may even lead to the down-regulation of COX-2 activity and decreased formation of proinflammatory eicosanoids. The exact mechanisms involved in stimulation of COX-2 activity by iNOS/NO are not yet understood (31). It is possible that peroxynitrite, the coupling product of NO and superoxide anion, acts as oxidant for the heme of COX-2 and activates the enzyme, as suggested by Marnett et al. (44). In addition to its stimulatory effects on COX-2 activity, NO or its metabolites may also affect tumor formation and/or progression by a number of other potential mechanisms. NO is an endothelial growth factor and specifically mediates the tumor vasculization to regulate blood flow (38). NO has also been found to directly or indirectly damage DNA, ultimately resulting in mutations, which may alter normal cellular processes (27). In addition, NO has been found to post-translationally modify a number of important cellular proteins, including p53, caspases, and DNA repair enzymes (28, 29). Either alone or in combination, these cellular alterations could contribute to protumorigenic phenotypes, including the formation of ACF, and ultimately to tumor formation.

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Chinthalapally V. Rao, Cooma Indranie, Barbara Simi, et al.


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