Prevention of NNK-induced Lung Tumorigenesis in A/J Mice by Acetylsalicylic Acid and NS3981

2 authors:

Nathalie Rioux
Certara

84 PUBLICATIONS   2,950 CITATIONS

SEE PROFILE

Andre Castonguay
Université Laval

133 PUBLICATIONS   4,788 CITATIONS

SEE PROFILE

Cancer Research

AACR

Prevention of NNK-induced Lung Tumorigenesis in A/J Mice by Acetylsalicylic Acid and NS-398

Nathalie Rioux and Andre Castonguay

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/58/23/5354

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Prevention of NNK-induced Lung Tumorigenesis in A/J Mice by Acetylsalicylic Acid and NS-398[1]

Nathalie Rioux and Andre Castonguay[2]

Laboratory of Cancer Etiology and Chemoprevention, Faculty of Pharmacy, Laval University, Quebec City, Canada, G1K 7P4

ABSTRACT

Acetylsalicylic acid (ASA) is known to prevent cancer development, but its mechanism of action remains unclear. In this study, we compared the efficacies of this nonspecific cyclooxygenase (COX) inhibitor with N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398), a specific COX-2 inhibitor. COX-2-specific inhibitors are less toxic than ASA. Lung tumorigenesis was induced in A/J mice by the administration of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the drinking water for 7 weeks (weeks 0 to +7). Groups of 25 A/J mice were fed ASA (588, 294, 147, or 73 mg/kg diet) before and throughout the assay (weeks −2 to +23). ASA at a dose of 588 mg/kg diet was the most effective because it reduced lung tumor multiplicity by 53%. The preventive effect of ASA increased with the dose, being of 32, 30, and 44% for 73, 147, and 294 mg/kg diet, respectively. NNK increased plasma prostaglandin E_2 (PGE_2) basal levels by 413%, whereas ASA attenuated this elevation in a dose-response manner ($r^2 = 0.99$). Plasma PGE_2 levels in ASA + NNK-treated mice correlate with the logarithm of the number of tumors ($r^2 = 0.99$). NS-398 inhibited lung tumor multiplicity by 34% and returned plasma PGE_2 to basal levels observed in untreated mice. Among the NNK-exposed mice, ASA and NS-398 treatment decreased the mean of the lung tumor volumes. Incubation of 82–132 and LM2 murine lung tumor cells with ASA or NS-398 decreased cell proliferation by 50% at concentrations higher than 100 μM. Incubations of NNK with COX-1 and -2 produced both activation and detoxification products by α-carbon hydroxylation and N-oxydation pathways, respectively. Bioactivation of NNK was more extensive by COX-2 than COX-1. Anti-COX-1 and -2, arachidonic acid, ASA, and NS-398 inhibited NNK bioactivation by COX-1 and -2 from 22–49%. Our data suggest that NNK is bioactivated by COX-2 in lung tissues and that COX-2-specific inhibitors might be promising chemopreventive agents.

INTRODUCTION

NSAIDs[3] have been shown to prevent tumorigenesis in humans and in laboratory animals (1, 2). In humans, death rates from colorectal, mammary, esophageal, and gastric cancer decreased with frequent ASA use (Fig. 1; Refs. 3–5). Duperron and Castonguay (6) showed that ASA reduced NNK-induced lung tumorigenesis by 60% in A/J mice. ASA, used at the usual recommended dose, showed a toxicity level that could outweigh the benefits of its cancer-preventive effect (7). The major adverse effect of ASA is gastrointestinal toxicity, which ranges from mild dyspepsia to bleeding, perforation, and even death (8). NSAIDs, including ASA, also induce several nephrological syndromes (9). The development of new drugs with less adverse effects would be a promising alternative to the use of presently available NSAIDs as chemopreventive agents.

The gastrointestinal toxicity of NSAIDs results from a nonspecific

inhibition of COX (also known as prostaglandin H synthase) by ASA. COX-1, the constitutive form, synthesizes prostacyclin, which is cytoprotective for the gastric mucosa (10). The second isoform, COX-2, is induced by inflammatory stimuli (11, 12). In humans, as in rodents, the expression of COX-2 is significantly higher in colorectal tumors than in normal mucosa (13–16). Furthermore, Oshima et al. (17) showed that COX-2 null mice had a dramatic reduction in the number and size of intestinal polyps, from which adenomas can develop. The study of Oshima et al. (17) provided the genetic evidence that COX-2 plays a key role in tumorigenesis. Selective inhibition of COX-2 is a promising approach to the prevention of lung carcinogenesis.

COX-1 and -2 are structurally distinct with 60% homology in humans (18). The development of specific COX-2 inhibitors leads to a second generation of NSAIDs, with significantly reduced adverse effects (19). Among those, NS-398 is nonulcerogenic at doses up to 1000 mg/kg body weight in rodents (Fig. 1; Refs. 20 and 21). Thus, COX-2 inhibitors could be given for extended periods of time in long-term prevention trials, which presents a considerable advantage over the administration of ASA. A slow structural transition of COX-2 is induced by NS-398 in a time- and concentration-dependent manner (22). Recently, NS-398 has been shown to reduce azoxymethane-induced aberrant crypt foci, which are considered colon preneoplastic lesions in rats (23–24). The preventive efficacies of second-generation NSAIDs against lung carcinogenesis have not been investigated.

ASA possesses both anti-inflammatory and analgesic properties. As described above, prevention of carcinogenesis has been associated with its anti-inflammatory activity (2). In contrast to ASA, acetaminophen (Fig. 1), an analgesic with no or negligible anti-inflammatory activity, was found not to be associated with decreased risk of any digestive tract cancer in epidemiological studies (4, 25). In this study, acetaminophen was included as a control in the mouse lung tumor assay to asses the contribution of the analgesic activity of NSAIDs to lung cancer prevention.

Lung cancer is the leading cause of cancer mortality for both men and women in North America (26). The nicotine-derived N-nitrosamine NNK, which is present in tobacco smoke, induced lung tumors in rodents and is most likely involved in lung carcinogenesis in smokers (27). Metabolic activation of NNK by cytochrome P450 is required for its carcinogenic activity (28). A possible activation of NNK by COX-1 and/or -2 has never been investigated. COX, by way of its peroxidase activity, catalyzes the oxidation of a wide range of xenobiotics, including chemical carcinogens, such as benzo(a)pyrene, benzidine, and 2-aminofluorene (29–31).

The aims of this study were: (a) to document the relationship between the dose and preventive efficacy of ASA, because we observed in a previous study that ASA was effective at a dose of 294 mg/kg diet (6); (b) to demonstrate, for the first time, the efficacy of a specific COX-2 inhibitor, NS-398, to prevent lung tumorigenesis in A/J mice, and to compare its prophylactic potential to ASA, a nonspecific COX inhibitor; (c) to investigate the effects of ASA and NS-398 on murine lung tumor cell proliferation; and (d) to determine whether COX-1 and/or -2 are implicated in the metabolic activation of NNK.
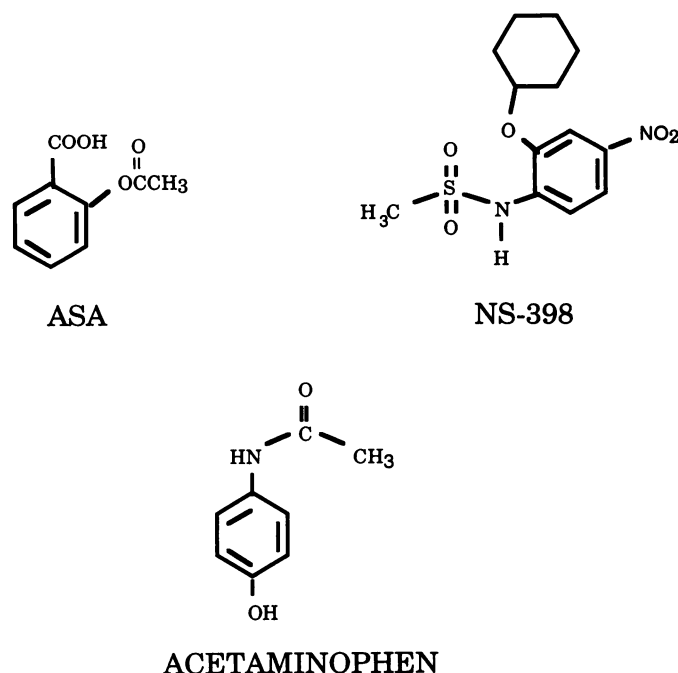
Fig. 1. Chemical structure of acetaminophen, ASA, and NS-398.

MATERIALS AND METHODS

Chemicals

NNK (99% pure, determined by TLC) and [5-^{3}H]NNK (99% pure, determined by HPLC) were purchased from Chemsyn Science Laboratories (Lenexa, KS). The synthesis of metabolites used as standard in HPLC analysis of NNK metabolism has been reported (32). Acetaminophen (99% pure), 2-acetamidophenol, ASA (99.5% pure), BSA, DMSO, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, hydrochlorothiazide, indo-methacin, 3,[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide, and NADP$^+$ were purchased from Sigma-Aldrich Canada (Oakville, Canada). COX-1 and -2 monoclonal antibodies (ovine COX-1 and -2), arachidonic acid, and NS-398 (98.5% pure) were supplied by Cayman Chemical Co. (Ann Arbor, MI). The standard [5, 6, 8, 11, 12, 14, 15-^{3}H(N)]PGE$_2$ (200 Ci/mmol) was purchased from NEN DuPont (Mississauga, ON, Canada). Antirat NADPH P-450 reductase serum was purchased from Gentest Corporation (Woburn, MA). DMEM, FBS, penicillin-streptomycin solution, and trypsin-EDTA were bought from Life Technologies, Inc. (Burlington, ON, Canada).

Lung Tumor Assay in A/J Mice

A/J female mice (18–22 g), 7 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). The animals were maintained under specific pathogen-free conditions and were housed under standardized conditions (five mice/cage; 22 ± 2°C; 28 ± 5% relative humidity; 12-h light-dark cycles). Animals were treated in accordance with approved institutional protocols and following guidelines of the Canadian Council of Animal Care. Stock solutions of NNK were prepared in distilled water (5 mg/ml) and diluted in tap water. The concentration of NNK at the beginning of the experiment was 65 μg/ml and was adjusted thereafter for each cage according to water consumption, which was monitored twice weekly for 7 weeks. AIN-76A powdered diet was purchased from Teklad Premier (Madison, WI) and used within a month. Chemopreventive agents were mixed with the diet in a V-blender for 1 h to obtain a homogeneous preparation. Diets were prepared weekly and stored in sealed containers in the dark at 4°C. Animals had access to the food and water ad libitum, and the powder feeders (Lab Product, Maywood, NJ) were cleaned and replenished with fresh diet twice a week. Diet consumption was monitored six times during the bioassay. Groups of 5 mice from the same cage were weighed weekly. Details of treatment with NNK and chemopreventive agents are included in Table 1. Group 1 (22 mice) received the diet without chemopreventive agents and was given tap water ad libitum. Groups 2–8 (25 mice) received NNK in the drinking water for 7 weeks (weeks 0 to +7). Group 2 was fed drug-free AIN-76A. Diets including chemopreventive agents were given to groups 3–8, starting 2 weeks before the treatment with NNK and continuing throughout the assay (weeks −2 to +23). Chemopreventive agents were given at nontoxic doses.

Sixteen weeks after NNK treatment (week +23), the mice were fasted overnight, anesthetized with a solution of ketamine-xylazine, killed by cervical dislocation, and necropsied. Lungs were fixed in Tellyesniczky's fixative for at least 7 days before counting the number of surface adenomas >1 mm. The diameters of the tumors were measured to determine their volume, using a dissecting microscope and a 10 mm/0.1 micrometer. The volumes of the round-shaped tumors were calculated as spherical volumes by the formula, $v = \pi d^3/6$, where "d" is the mean of at least two diameter measurements. Ten tumors/group were embedded in paraffin and stained for histopathological examination. Stomachs were fixed in situ with 0.5 ml of 10% formalin, excised, and stored in formalin. Papillomas >1 mm were counted.

Analysis of Drug Stability in Diets

ASA. Diet samples containing 294 mg of ASA/kg of diet were recovered from feeders after a 4-day period of feeding. Samples (2 g) of diet were extracted with 20 ml of a 9:1 mixture of diethyl ether:0.01N HCl. A 100-μl aliquot of hydrochlorothiazide solution (100 μg/ml methanol) was added as an

Table 1 *Effects of chemopreventive agents on lung tumorigenesis in A/J mice*

				Lung tumor			
Group no.	NNK mg/mouse[a] (mean ± SD)	Chemopreventive agent[b]	Dose of agent mg/kg diet (mmol/kg BW[c]/day)	Multiplicity[d] (mean ± SE)	Incidence[e]	Volume[f] (mm^3)	Plasma PGE$_2$ pg/ml (mean ± SE)
1 (Negative)	None	None	None	0.3 ± 0.1	6/22	None	49 ± 4
2 (Positive)	9.09 ± 0.02	None	None	8.7 ± 1.2	29/29	2.2 ± 0.2	260 ± 68[g]
3	9.11 ± 0.05	ASA	588 (4.90)	4.1 ± 0.7[h]	24/24	0.7 ± 0.5[i]	42 ± 5
4	9.10 ± 0.05	ASA	294 (2.45)	4.8 ± 0.5[h]	24/24	1.4 ± 0.1[j]	120 ± 35[g]
5	9.09 ± 0.03	ASA	147 (1.22)	6.0 ± 1.1	24/24	1.6 ± 0.2[j]	206 ± 30[g]
6	9.08 ± 0.04	ASA	73 (0.61)	5.9 ± 0.5	25/25	1.4 ± 0.1[j]	183 ± 50[g]
7	9.10 ± 0.07	Acetaminophen	1520 (15.1)	6.5 ± 0.8	25/25	1.5 ± 0.1[j]	260 ± 77[g]
8	9.08 ± 0.04	NS-398	7 (0.03)	5.7 ± 0.9[k]	25/25	1.1 ± 0.1[i]	49 ± 8

[a] NNK was given in the drinking water from week 0 to +7. Total dose was estimated to be 2.2 mmol/kg BW.
[b] Chemopreventive agents were given in the diet from week −2 to sacrifice (week +23).
[c] BW, body weight.
[d] All groups are statistically different from the negative control (group 1) at $P < 0.005$.
[e] The incidence is the number of mice bearing at least one tumor on the number of mice in the group. Group 1 is statistically different from all other groups (χ^2 analysis, $P < 0.001$).
[f] Determined from 45 to 96 tumors located in 15 to 25 mice/group, statistically different from group 2, post-hoc ANOVA.
[g] Statistically different from group 1, post-hoc ANOVA ($P < 0.05$).
[h] Statistically different from positive control, post-hoc ANOVA ($P < 0.01$).
[i] $P < 0.001$.
[j] $P < 0.01$.
[k] Statistically different from positive control, post-hoc ANOVA ($P < 0.05$).

5355

Table 2 *Metabolism of NNK by mouse lung microsomes and/or ovine COXs*

Incubation variables				NNK metabolites[a]		
Incubation no.	Microsome (mg protein)	COX	Inhibitors	α-carbon hydroxylation (%)[b]	N-oxydation (%)	NNK+NNAL (%)[c]
1	None	None	None	0.9	1.1	97.9
2	0.5	None	None	8.1 ± 0.6	3.9 ± 1.3	87.9 ± 1.3
3	0.5	COX-1	None	8.8 ± 0.4	5.9 ± 0.8	85.2 ± 0.8
4	None	COX-1	None	5.5 ± 0.7	3.9 ± 0.3	90.5 ± 0.8
5	0.5	COX-1	Arachidonic acid	3.4 ± 0.1[d]	4.0 ± 1.3	92.5 ± 0.9
6	None	COX-1	ASA	3.9 ± 0.6[d]	2.5 ± 0.5[d]	93.3 ± 0.9
7	0.5	COX-2	None	9.9 ± 1.0	7.1 ± 0.6[e]	82.8 ± 0.7
8	None	COX-2	None	8.9 ± 0.3	6.1 ± 0.3[e]	84.8 ± 0.5
9	0.5	COX-2	Arachidonic acid	7.1 ± 1.0	5.6 ± 0.2	87.2 ± 1.2
10	None	COX-2	ASA	6.7 ± 0.4[f]	4.6 ± 0.1[f]	88.6 ± 0.3[f]
11	None	COX-2	NS-398	6.3 ± 0.6[f]	4.8 ± 0.2[f]	88.5 ± 0.9[f]

[a] This metabolic pathways has been detailed by Jorquera *et al.* (36).
[b] The percentage of metabolites on NNK and NNAL. Mean of three determinations ± SE.
[c] Percentage from initial amount of NNK.
[d] Statistically different from group 3, post-hoc ANOVA (*P* < 0.05).
[e] Statistically different from group 2.
[f] Statistically different from group 8, post-hoc ANOVA (*P* < 0.05).

internal standard to a 2-ml aliquot of supernatant. Samples were evaporated to dryness and resuspended in 200 μl of methanol. This extraction was repeated once. ASA stability was determined by reverse phase HPLC on a C-18 μBondapak column (Waters Associates, Milford, MA). The mobile phase consists of 95% methanol:0.02 M potassium phosphate buffer (pH 5.0; v/v, 40:60). Elution at a flow rate of 1 ml/min was monitored at 254 nm. Hydrochlorathiazide eluted at 3 min, and ASA eluted at 6 min. The limit of detection of ASA was 1 μg/ml. Concentrations were calculated from linear regression curves, relating peak areas to ASA and internal standard concentrations.

Acetaminophen. Stability of acetaminophen was determined as described for ASA with the following modifications: 2-acetamidophenol (1 mg/ml) was added as an internal standard. The HPLC mobile phase consisted of 0.1 M potassium dihydrogen phosphate:glacial acetic acid:propan-2-ol (v/v/v, 100: 0.1:0.75). Elution at a flow rate of 1.5 ml/min was monitored at 254 nm. Acetaminophen eluted at 18 min, and 2-acetamidophenol eluted at 24 min.

NS-398. Stability of NS-398 was determined as described for ASA with the following modifications: sulindac (20 μg/ml) was added as an internal standard. The HPLC mobile phase consisted of 0.1 M potassium phosphate:methanol (v/v, 1:1; pH 7.0). Elution at a flow rate of 1 ml/min was monitored at 280 nm. Sulindac eluted at 4.5 min, and NS-398 eluted at 12 min.

Assay of Plasma PGE₂

At sacrifice, blood was collected from the mice used in the lung tumor bioassay by cardiac puncture, and plasma PGE_2 was extracted and measured as described previously (33–35) using a competitive PGE_2-monoclonal enzyme immunoassay (Cayman Chemical Co.).

Preparation of Microsomes

Lung microsomes from A/J female mice were prepared by differential centrifugation, as described previously (36). Protein content, as determined by the method of Lowry *et al.* (37), using BSA as a standard, was 1.95 mg of protein/ml of microsomes.

## Assay of NNK Metabolism by Microsomal Proteins and COX-1 and -2

Enzymatic incubations were performed as described by Smith *et al.* (38) with some minor modifications. The incubation mixture consisted of 5 mM glucose-6-phosphate, 1.52 units of glucose-6-phosphate dehydrogenase, 1 mM NADP⁺, 1 mM EDTA, 3 mM $MgCl_2$, 10 μM (13 μCi) of [5-³H]NNK, and 100 mM sodium phosphate buffer (pH 7.4) in a total volume of 800 μl. After incubation at 37°C for 10 min, the reaction was initiated by the addition of 0.5 mg of lung microsomal proteins or 1,000 units of COX-1 or -2 (20 μg of COX-1 and 105 μg of COX-2). The reaction was incubated for 30 min at 37°C and stopped by the addition of 200 μl of 25% zinc sulfate and 200 μl of saturated barium hydroxide. Some incubations were carried out with arachidonic acid, ASA or NS-398 (100 μM; Table 2). The drugs were shown to be stable under these experimental conditions. An incubation was also performed after the preincubation of COX-2 with 50 μl of antirat NADPH P-450 reductase serum for 30 min at room temperature. A control incubation was done with untreated rat serum. In Table 3, lung microsomes were preincubated with COX-1 and -2 monoclonal antibodies, 10 μg each, for 15 min at room temperature. Precipitated samples were centrifuged at 14,000 × g for 30 min and filtered through a 0.45-μm Millex-LCR (Millipore Corporation, Beford, MA). The filtrate (500 μl) and 7 μl of the standard mixture of metabolites were coinjected into a reversed-phase HPLC system, using a Spherisorb ODS 2 5-μm column (Jones Chromatography Inc., Columbus, OH). NNK and its metabolites were eluted with a pH-6.0 sodium acetate buffer and methanol, as described previously (36, 39). The elution was monitored at 254 nm, and 1-ml fractions were collected. Scintisafe Plus (4 ml; Fisher Scientific, Montreal, QC, Canada) were added to each fraction, and the radioactivity was measured by liquid scintillation spectroscopy. Recovery of total radioactivity during the HPLC analysis was >75%.

Table 3 *Metabolism of NNK by mouse lung microsomes in presence of the COX antibody*

Incubation variables			NNK metabolites[a]		
Incubation no.	Microsome (mg protein)	Antibody	α-carbon hydroxylation (%)	N-oxydation (%)	NNK+NNAL (%)
A	0.5	None	14.2 ± 2.1	5.4 ± 0.3	78.6 ± 0.7
B	0.5	Anti-COX-1 and -2	9.6 ± 0.9	5.2 ± 0.1	85.2 ± 0.9[b]

[a] The percentage of metabolites on NNK and NNAL from the initial amount of NNK. Mean of three determinations ± SE. This metabolic pathways has been detailed by Jorquera *et al.* (36).
[b] Statistically different from no. A, post-hoc ANOVA, *P* < 0.05.

5356

Fig. 2. Average body weight of the A/J mice during the lung tumor assay. Each point corresponds to the mean of 5 groups of 5 mice with a SD <10%. Numbers correspond to the groups in Table 1: *1*, negative control; *2*, NNK only; *4*, ASA + NNK; *7*, acetaminophen + NNK; *8*, NS-398 + NNK. *, groups 2, 4, 7 and 8 were statistically different from group 1 during carcinogen treatment (Student's t test, $P < 0.05$).
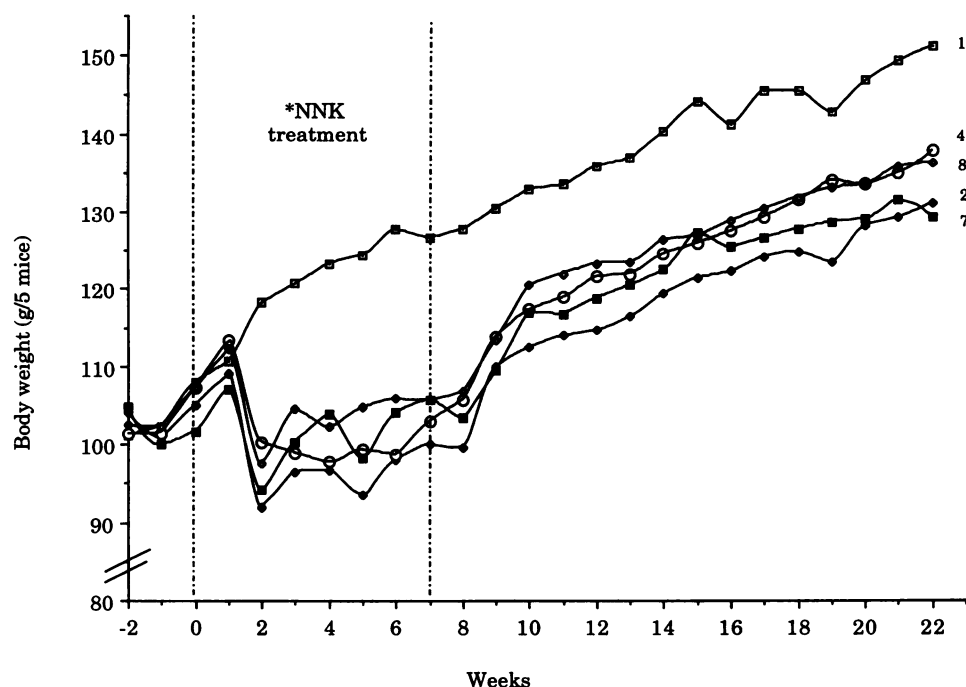
Cell Lines

Murine lung cells, 82–132, originating from a type II solid carcinoma, were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin (40). The LM2 murine lung epithelial cells, derived from a papillary tumor, were cultured in MEM α supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were grown in 25-cm^3 plastic dishes and split every three days using trypsin-EDTA. Cells were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2.

Cell Proliferation Assay

Lung tumor cells were cultured on 96-well plates, at an initial concentration of 1×10^4 cells/well with or without the chemopreventive agents. Chemopreventive agents were dissolved in DMSO. The concentration of DMSO in all wells, including control wells, was 2% and shown to be nontoxic. After a 72-h incubation at 37°C, 25 μl of a sterile solution of 3,[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide in PBS (5 mg/ml) were added to each well, and incubation was continued for 2 h at 37°C. The cells were lysed with 100 μl of the extraction buffer, which consists of 20% w/v of SDS dissolved in a 50% solution of N'-N'-dimethyl formamide, and water was adjusted at pH 4.7. Optical densities at 570 nm were measured after an 18-h incubation at 37°C (41).

Statistical Analysis

Multiplicity and volumes of lung tumor adenomas, PGE_2 levels, and NNK metabolism were compared using post-hoc ANOVAs. Incidences of lung adenomas were compared using χ^2 analyses. Differences between samples were considered statistically significant at the $P < 0.05$ level.

RESULTS

Lung Tumor Assay. NNK given at a cumulative dose of 9.08–9.11 mg/mouse, during 7 weeks, prevented normal body weight gain (Fig. 2). Body weights of NNK-treated mice sharply increased after cessation of NNK treatment, but never reached that of untreated mice. Body weights of mice treated with NNK, with or without chemopreventive agents, were not statistically different from untreated mice (group 1) at the time of sacrifice. Chemopreventive agents were given at nontoxic doses. Food consumption by mice treated with NNK, with or without chemopreventive agents, was not statistically different

from consumption by untreated mice and was ranging from 2.38–2.57 g/mouse/day. At the time of sacrifice, no gross pathological change related to toxicity was observed in the livers, kidneys, stomachs, intestines, or lungs of mice fed the NNK + chemopreventive agent diets. Screening of the mouse colony indicated no viral or bacterial infection at the end of the lung tumor bioassay. One mouse from groups 3, 4, and 5 was sacrificed before the end of the assay due to diarrhea, dehydration, or extra-abdominal tumor and was eliminated from the study.

The effects of chemopreventive agent treatments on lung tumorigenesis are shown in Table 1. As expected, untreated mice (group 1) had a small incidence of "spontaneous" tumors; only 6 of 22 mice were carrying one or two lung tumors. Treatment with NNK (group 2) induced 8.7 tumors/mouse, identified as pneumocyte type-II-derived solid tumors, with an average volume of $2.2 \pm 0.2 \text{ mm}^3$ (Table 1). Feeding ASA, 73–588 mg/kg diet, throughout the study (weeks −2 to
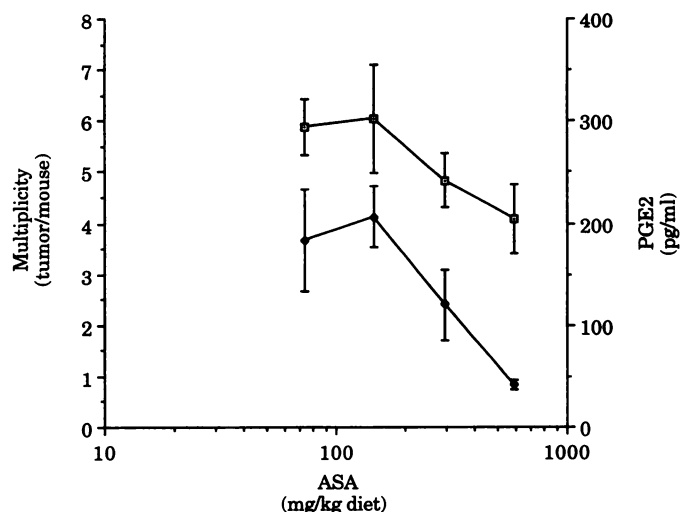

Fig. 3. Dose-response effect of ASA on lung tumorigenesis and plasma PGE_2 levels. The doses of ASA are 588, 294, 147, and 73 mg/kg diet. The r^2 is 0.90 for lung tumor multiplicity (□) and 0.98 for plasma levels of PGE_2 (◆).

5357

+23) reduced the lung tumor multiplicity in a dose-dependent manner from 30–53% with a r^2 of 0.90 (Fig. 3). NS-398 reduced lung tumor multiplicity by 34% ($P < 0.05$). ASA treatment reduced lung tumor volume in a dose-dependent manner, from 27–68% ($r^2 = 0.8$), whereas NS-398 treatment caused a 50% decrease of the mean tumor volume ($P < 0.001$). As expected, lung tumor multiplicity inhibition by acetaminophen treatment was not statistically significant ($P > 0.05$). The lung tumor multiplicity was confirmed by morphological identification. The incidence of gastric papillomas was low and not statisticaly different among groups (data not shown).

Stability of Drugs in the Diet. The chemopreventive agents mixed with AIN-76A diet were relatively stable under the feeding conditions. We recovered 99% of NS-398 and 98% of acetaminophen from the diets under feeding conditions. Although 93% of the ASA was recovered, a significant variation between samples (up to 20%) was observed (data not shown).

Inhibition of PGE$_2$ Synthesis. More than 85% of PGE$_2$ was recovered from the plasma. The PGE$_2$ level in untreated mice was 49 ± 4 pg/ml (Table 1). NNK treatment raised this level to 260 ± 68 pg/ml ($P < 0.05$). As expected, acetaminophen had no effect on plasma PGE$_2$ levels, whereas ASA treatment lowered the plasma PGE$_2$ level from 206 ± 30 pg/ml to 42 ± 35 pg/ml. Plasma PGE$_2$ in NS-398-treated mice and in untreated mice were similar. In ASA-treated mice, plasma PGE$_2$ levels and lung tumor multiplicity were correlated with a r^2 of 0.98 (Fig. 3).

Metabolism of NNK by Microsomal Lung Proteins and COX-1 and -2. NNK is bioactivated by hydroxylation of the carbons adjacent (α-carbon) to the N-nitroso nitrogen, producing hydroxy acid, keto acid, diol, and keto alcohol. NNK-N-oxide and NNAL-N-oxide result from N-oxidation of NNK and NNAL, respectively (28). A small amount of NNK is spontaneously transformed into its various products in the metabolism assay (incubation no. 1; Table 2). As expected, A/J mouse lung microsomes metabolized [5-^{3}H]NNK by α-carbon hydroxylation (8.1%) and N-oxidation (3.9%; incubation no. 2). In the absence of microsomal proteins, COX-1 metabolized 9.5% of NNK, via α-carbon hydroxylation (5.5%) and N-oxydation (3.9%; incubation no. 4). Arachidonic acid and ASA added to COX-1 and microsomal proteins inhibited NNK bioactivation by 49.3% and 29.5%, respectively ($P < 0.05$; incubations no. 5 and 6). COX-2 metabolized 15.2% of the initial amount of NNK, and the addition of microsomal proteins raised this level to 17.2% (incubations no. 7 and 8). As expected, preincubation of COX-2 with anti-P450 reductase did not significantly reduced NNK metabolism (data not shown). Arachidonic acid, ASA and NS-398 inhibited COX-2-dependent NNK bioactivation by 25.6%, 25.0%, and 24.3% ($P < 0.05$), respectively. The preincubation of lung microsomal proteins with COX-1 and -2 monoclonal antibodies resulted in a 22.0% inhibition of NNK bioactivation (Table 3).

Inhibition of Cell Proliferation by ASA and NS-398. We evaluated the effects of ASA and NS-398 on proliferation of both 82–132 and LM2 cell lines (Fig. 4). Growth of the 82–132 cell line was inhibited with an EC$_{50}$ of 140 μM for both ASA and NS-398. With the LM2 cell line, EC$_{50}$ was over 200 μM for ASA and 115 μM for NS-398. At a 25-μM concentration of either agent, growth inhibition was <5%.

DISCUSSION

Arachidonic acid is metabolized to prostaglandins, prostacyclins, and thromboxanes by cyclooxygenases. Two forms of the enzyme, COX-1 and -2, have been identified as the constitutive and the inducible forms, respectively. The preventive potential of ASA, a nonspecific COX inhibitor, against carcinogenesis is well docu-
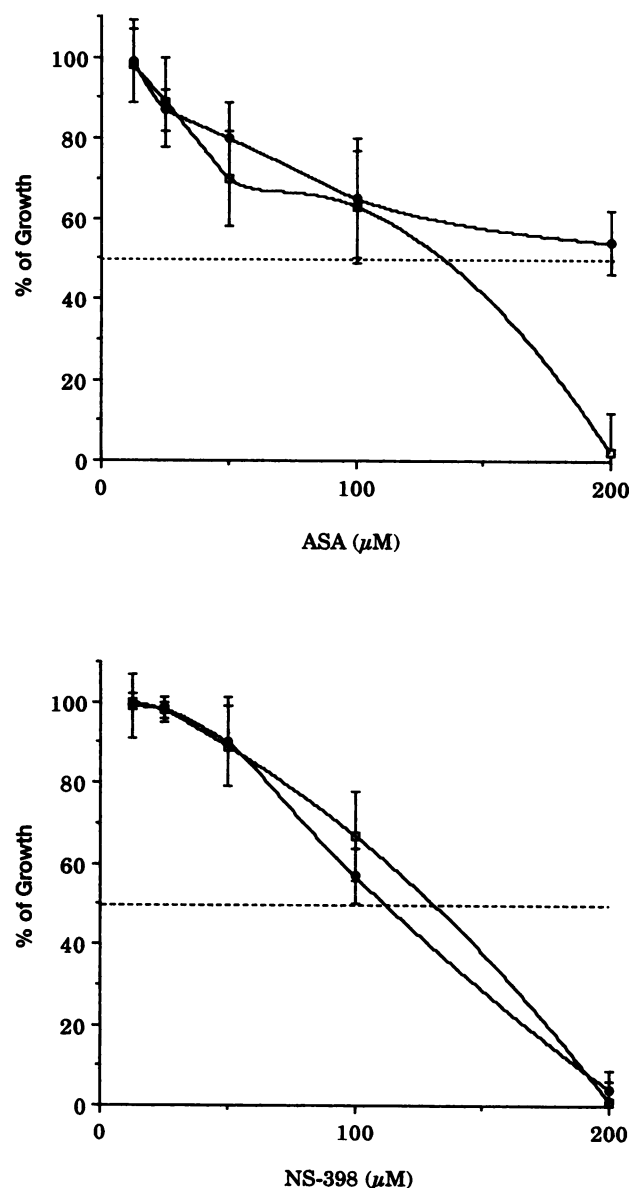

Fig. 4. Inhibition of proliferation of murine lung tumor cells by ASA and NS-398. Each point corresponds to the mean of three determinations $\pm$ SE. □, 82–132 cell line; ●, LM2 cell line.

mented, but the preventive efficacies of COX-2-specific inhibitors have never been investigated (2). These specific inhibitors are far less toxic than other conventional NSAIDs, including ASA, thus they could be used as an alternative to minimize toxicity in future prevention trials (1, 20). In this study, we observed that ASA and the COX-2-specific inhibitor NS-398 reduce lung tumor multiplicity and volumes in A/J mice. The present study is the first to demonstrate that both COX isoenzymes are able to bioactivate NNK. Our results suggest that COX-2 inhibitors are efficient cancer preventive agents of lung tumorigenesis.

The first aim of this study was to determine whether the preventive efficacy of ASA against lung tumorigenesis was dose-related. Duperron and Castonguay (6) had previously shown that a dose of 294 mg/kg diet of ASA reduces lung tumor multiplicity by 60% in NNK-treated mice. Female A/J mice were exposed to low doses of NNK in drinking water, with or without chemopreventive agents, for an extended period of time to mimic the exposure of smokers to this tobacco-specific nitrosamine. As shown in Table 1, ASA reduced

NNK-induced lung tumorigenesis in a dose-dependent manner. A similar effect, proportional to the logarithm of the dose, was previously observed with another NSAID, sulindac (42). In this study, ASA, at a dose of 588 mg/kg diet, inhibited lung tumor multiplicity by 53% ($P < 0.01$). The doses of NSAIDs used in this study are comparable with the maximal recommended daily doses for humans. In this study, we used ASA at doses ranging from 7.9–63.4 mg/kg body weight, whereas the recommended maximal antipyretic and anti-inflammatory doses are 55.2 and 83.6 mg/kg body weight, respectively. The mechanism of cancer prevention by ASA seems to be by nonspecific inhibition of COXs (2, 19, 33). ASA irreversibly inhibits COX-1 and -2 by acetylation, with a higher activity against COX-1 (19). COX-2 mRNA has been shown to be elevated in a number of human and animal tumors (13, 43–45). Furthermore, COX-2 levels are induced at an early stage of tumorigenesis, as shown in the murine model of familial adenomatous polyposis developed by Oshima et al. (17). In this study, we observed a 5-fold increase of the plasma PGE_2 level, one of the principal products of COX, in NNK-treated mice. This increase of the plasma PGE_2 level was totally inhibited by the 588 mg/kg diet dose of ASA, but as previously mentioned, lung tumor multiplicity was decreased by only 53%. These results lead us to conclude that a 50–60% reduction of lung tumor multiplicity is the maximum level of inhibition achievable by nontoxic doses of ASA in mice. Our results are in line with those of Oshima et al. (17) who observed a partial, but not complete, inhibition of intestinal polyposis in COX-2 knockout mice. Furthermore, an elevation in ASA dosage, over 4–6 tablets/week, did not decrease the risk of colorectal cancer in humans, suggesting a saturation point for ASA-preventive efficacy (5). Mechanisms other than COX's inhibitions might be involved in cancer prevention by ASA.

Toxic effects of ASA are a major obstacle in the development of this agent as a chemopreventive agent (1). ASA and indomethacin are two NSAIDs that cause the most damage to the stomach (46). Long-term administration of ASA is toxic to the upper gastrointestinal system and induces nephrological and hepatotoxic syndromes (8, 9, 47). An alternative to ASA cancer prevention would be to use a COX-2-specific inhibitor, such as NS-398, which causes minimal stomach lesions (21). In this study, we show for the first time that NS-398 inhibits lung tumorigenesis. As shown in Table 1, NS-398 treatment reduced NNK-induced tumorigenesis by 34% ($P < 0.05$). Recently, Kawabata et al. (23) observed a reduction of azoxymethane-induced aberrant crypt foci, which are considered colon preneoplastic lesions in NS-398-treated rats (24). Thus, the cancer-preventive efficacy of NS-398 is not limited to the lungs. The observation of NS-398 antitumorigenic activity is of great interest because COX-2-specific inhibitors could be administered to humans for an extended period of time without the major toxic effects characteristic of NSAID treatment.

As shown in Table 1, administration of ASA and NS-398 to NNK-treated mice reduced tumor volume. Herein, we investigate the effects of NS-398 and ASA on murine lung cell proliferation. As shown in Fig. 4, NS-398 and ASA inhibited 82–132 and LM2 lung cell proliferation only at concentrations higher than those achievable in mice plasma. ASA was shown to be a weak growth inhibitor of the HT-29 colon cancer line (48). The inhibitory concentration of ASA and NS-398 could not be obtained in blood after administration of nontoxic doses of either agents. These results lead us to conclude that the antiproliferative action of NS-398 and ASA could contribute to some extent to the inhibition of lung tumor development.

One aim of this study was to determine whether COX-1 and -2 could bioactivate NNK with their peroxidase activity (49). In this study, we observed a bioactivation of NNK by COX-1 and -2, in the presence or absence of microsomal proteins. As shown in Table 2,

COX-2 metabolized NNK more effectively than COX-1. NNK was metabolized via both α-carbon hydroxylation and N-oxidation pathways. Inhibition of NNK metabolism by arachidonic acid, a natural substrate for COX acting as a competitive inhibitor, ASA and NS-398 were above the expected values. We were surprised to observe some affinity of NNK for COX, which could explain the weak inhibitory potency of these compounds. This might also reflect the smaller inhibitory potential of NSAIDs on peroxidase activity compared with cyclooxygenase activity (50). We observed an inhibition of NNK bioactivation in mouse lung microsomes by COX-1 and -2 antibodies (Table 3). COXs, especially COX-2, may be important for NNK activation in extrahepatic tissues, and could work in parallel with the P450 pathway of NNK metabolism. Preliminary data showed that human COX, expressed in the U-937 cell line, activated NNK. We concluded that inhibition of COX-dependent NNK activation by ASA and NS-398 in lung tissue is a possible mechanism of action of these drugs in lung cancer prevention.

The analgesic acetaminophen was included in this mouse lung tumor assay to determine whether the analgesic activity of ASA plays a role in the prevention of tumorigenesis. As expected, acetaminophen did not lower the PGE_2 plasma level in NNK-treated mice. Unexpectedly, acetaminophen decreased the number of lung tumors by 25% (Table 1; statistically not significant). Yao et al. (51) observed that acetaminophen treatment of mice given NNK inhibited the COX-2 expression in lung tissus by 16%. At the high dose of acetaminophen used in this study, a slight PGE_2 inhibition has been observed in addition to an analgesic activity (52). Acetaminophen has also been shown to have a weak anti-inflammatory activity at therapeutic doses (53). The small inhibition by acetaminophen observed in this study could be related to an increase in cell death: high concentrations of acetaminophen up-regulated cytosolic Ca^{2+} leading to necrosis (54). We concluded that the not statistically significant inhibition of lung tumorigenesis observed in acetaminophen-treated mice is most likely unrelated to its analgesic activity.

In summary, ASA inhibited lung tumor multiplicity in a dose-dependent manner. For the first time, NS-398 was also shown to inhibit lung tumorigenesis. This agent, being far less toxic, is a good alternative to long-term ASA administration. In addition, we observed that NNK is bioactivated by both COX-1 and -2. We believe that COX-2 contributes to NNK bioactivation in extrahepatic tissues. The mechanism of action of ASA and NS-398 seems to be an inhibition of both PGE_2 synthesis and COX's peroxidase-mediated NNK bioactivation. In short, our results suggest that the nontoxic COX-2-specific inhibitors are promising chemopreventive agents.

ACKNOWLEDGMENTS

REFERENCES

1. IARC. Handbooks of Cancer Prevention. Nonsteroidal Anti-inflammatory Drugs. IARC Sci. Publ., 1: 15–39, 1997.
2. Marnett, L. J. Aspirin and the potential role of prostaglandins in colon cancer. Cancer Res., 52: 5575–5589, 1992.
3. Harris, R. E., Namboodiri, K. K., and Farrar, W. B. Nonsteroidal anti-inflammatory drugs and breast cancer. Epidemiology, 7: 203–205, 1996.
4. Thun, M., Namboodiri, M., Calle, E., Flanders, W., and Heath, C. Aspirin use and risk of fatal cancer. Cancer Res., 53: 1322–1327, 1993.
5. Giovannucci, E., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Ascherio, A., and Willett, W. C. Aspirin use and risk for colorectal cancer and adenoma in male health professionals. Ann. Intern. Med., 121: 241–246, 1994.
6. Duperron, C., and Castonguay, A. Chemoprevention efficacies of aspirin and sulindac against lung tumorigenesis in A/J mice. Carcinogenesis (Lond.), 18: 1001–1006, 1997.

7. Trujillo, M. A., Garewal, H. S., and Sampliner, R. E. Nonsteroidal anti-inflammatory agents in chemoprevention of colorectal cancer. At what cost? Dig. Dis. Sci., 39: 2260–2266, 1994.

8. Gabriel, S. E., Jaakkimainon, L., and Bombardier, C. Risk for serious gastrointestinal complication related to use of nonsteroidal anti-inflammatory drugs. Ann. Intern. Med., 115: 787–796, 1991.

9. Clive, D. M., and Stoff, J. S. Renal syndromes associated with non-steroidal anti-inflammatory drugs. N. Engl. J. Med., 310: 563–572, 1984.

10. Whittle, B. J. R., Higgs, G. A., Eakins, K. E., Moncada, S., and Vane, J. R. Selective inhibition of prostaglandin production in inflammatory exudates and gastric mucosa. Nature (Lond.), 284: 271–273, 1980.

11. O'Bannion, M. K., Winn, V. D., and Yung, D. A. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. Proc. Natl. Acad. Sci. USA, 89: 4888–4892, 1992.

12. Hempel, S. L., Monik, M. M., and Hunnighake, G. W. Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. J. Clin. Invest., 93: 391–396, 1994.

13. Eberhart, G. E., Coffey, R. J., Radhika, A., Giardello, F. M., Ferrenbach, S., and DuBois, R. N. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology, 107: 1183–1188, 1994.

14. Yoshimi, N., Ino, N., Suzui, M., Tanaka, T., Nakashima, S., Nakamura, M., Nozawa, Y., and Mori, H. The mRNA overexpression of inflammatory enzymes, phospholipase A$_2$ and cyclooxygenase, in the large bowel mucosa and neoplasms of F344 rats treated with naturally occuring carcinogen, 1-hydroxyanthraquinone. Cancer Lett., 97: 75–82, 1995.

15. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res., 55: 3785–3789, 1995.

16. Boolbol, S. K., Dannenberg, A. J., Chadburn, A., Martucci, C., Guo, X., Ramonetti, J. T., Abreu-Goris, M., Newmark, H. L., Lipkin, M. L., DeCosse, J. J., and Bertagnolli, M. M. Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. Cancer Res., 56: 2556–2560, 1996.

17. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. Suppression of intestinal polyposis in APC$^{\Delta716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell, 87: 803–809, 1996.

18. Jones, D. A., Carlton, D. P., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines. J. Biol. Chem., 268: 9049–9054, 1993.

19. Vane, J. R. Towards a better aspirin. Nature (Lond.), 367: 215–216, 1994.

20. Arai, I., Hamasaka, Y., Futaki, N., Takahashi, S., Yoshikawa, K., Higuchi, S., and Otomo, S. Effect of NS-398, a new nonsteroidal anti-inflammatory agent, on gastric ulceration and acid secretion in rats. Res. Commun. Chem. Pathol. Pharmacol., 81: 259–270, 1993.

21. Futaki, N., Yoshikawa, K., Hamasaka, Y., Arai, I., Higuchi, S., Iizuka, H., and Otomo, S. NS-398, a novel non-steroidal anti-inflammatory drug with potent analgesic and antipyretic effects, which causes minimal stomach lesions. Gen. Pharmacol., 24: 105–110, 1993.

22. Copeland, R. A., Williams, J. M., Giannaras, J., Nurnberg, S., Covington, M., Pinto, D., Pick, S., and Trzaskos, J. M. Mechanism of selective inhibition of the inducible form of prostaglandin G/H synthase. Proc. Natl. Acad. Sci. USA, 91: 11202–11206, 1994.

23. Kawabata, K., Yoshimi, N., Hirose, Y., Hara, A., and Mori, H. Reduction of azoxymethane-induced aberrant crypt foci by a selective cyclooxygenase-2 inhibitor, NS-398 in F344 rats. Proc. Am. Assoc. Cancer Res., 38: 210, 1997.

24. Pretlow, T. P., O'Riordan, M. A., Pretlow, T. G., and Stellato, T. A. Aberrant crypts in human colonic mucosa: putative preneoplastic lesions. J. Cell. Biochem., 16G: 55X–62X, 1992.

25. Peleg, I. I., Maibach, H. T., Brown, S. H., and Wilcox, C. M. Aspirin and nonsteroidal anti-inflammatory drug use and the risk of subsequent colorectal cancer. Arch. Intern. Med., 154: 394–399, 1994.

26. Evans, W. K., Will, B. P., Berthelot, J-M., and Wolfson, M. C. The cost of managing lung cancer in Canada. Oncology, 9: 147–153, 1995.

27. Hoffmann, D., Rivenson, A., and Hecht, S. S. The biological significance of tobacco-specific N-nitrosamines: smoking and adenocarcinoma of the lung. Crit. Rev. Toxicol., 26: 199–211, 1996.

28. Hecht, S. S. Metabolic activation and detoxification of tobacco-specific nitrosamines–a model for cancer prevention strategies. Drug Metab. Rev., 26: 373–390, 1994.

29. Marnett, L. J., Reed, G. A., and Dennison, D. J. Prostaglandin synthetase dependent activation of 7,8-dihydroxy-benzo(a)pyrene to mutagenic derivatives. Biochem. Biopys. Res. Commun., 82: 210–216, 1978.

30. Robertson, I. G. C., Sivarajah, K., Eling, T. E., and Zeiger, E. Activation of some aromatic amines to mutagenic products by prostaglandin endoperoxide synthase. Cancer Res., 43: 476–480, 1983.

31. Sarkar, F. H., Radcliff, G., Callewaert, D. M., Marnett, L. J., Eling, T., Daston, D. S., and Caspary, W. J. Mutagenic response of mouse lymphoma cells after activation of benzidine and 2-aminofluorene with purified prostaglandin H synthase. Mutat. Res., 242: 319–328, 1990.

32. Hecht, S. S., Young, R., and Chen, C. B. Metabolism in the F344 rat of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen. Cancer Res., 40: 4144–4150, 1980.

33. Rioux, N., and Castonguay, A. Recovery from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induce immunosuppression in A/J mice by treatment with nonsteroidal anti-inflammatory drugs. J. Natl. Cancer Inst., 89: 874–880, 1997.

34. Powell, W. S. Rapid extraction of arachidonic acid metabolites from biological samples using octadecylsilyl silica. Methods Enzymol., 86: 467–477, 1982.

35. Pradelles, P., Grassi, J., and Maclowf, J. Enzyme immunoassays of eicosanoids using acetylcholinesterase. Methods Enzymol., 187: 24–34, 1990.

36. Jorquera, R., Castonguay, A., and Schuller, H. M. Effects of pregnancy and ethanol treatment on the metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by hamster liver and lung microsomes. Drug Metab. Dispos., 20: 510–517, 1992.

37. Lowry, O. H., Rosenbrough, N. J., Farr, L., and Randall, R. J. Protein measurements with the Folin phenol reagent. J. Biol. Chem., 193: 265–275, 1951.

38. Smith, T. J., Guo, Z., Thomas, P. E., Chung, F-L., Morse, M. A., Elkind, K., and Yang, C. S. Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in mouse lung microsomes and its inhibition by isothiocyanates. Cancer Res., 50: 6817–6822, 1990.

39. Castonguay, A., Lin, D., Stoner, G. D., Radok, P., Furaya, S. S., Hecht, S. S., Schut, A. G., and Klauning, J. E. Comparative carcinogenecity in A/J mice and metabolism by cultured mouse peripheral lung of N'-nitrosonornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and their analogues. Cancer Res., 43: 1223–1229, 1983.

40. Pan, Y. H. L., Nuzum, E., Hanson, L. A., and Beer, D. G. Ki-ras activation and expression in transformed mouse lung cell lines. Mol. Carcinog., 3: 279–286, 1990.

41. Hansen, M. B., Nielson, S. E., and Berg, K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods, 119: 203–210, 1989.

42. Castonguay, A., and Rioux, N. Inhibition of lung tumourigenesis by sulindac: comparison of two experimental protocols. Carcinogenesis (Lond.), 18: 491–496, 1997.

43. Liu, N., and Rose, D. P. Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines. Cancer Res., 56: 5125–5127, 1996.

44. Williams, C. S., Luongo, C., Radhika, A., Zhang, T., Lamps, L. W., Nanney, L. B., Beauchamp, R. D., and DuBois, R. N. Elevated cyclooxygenase-2 levels in Min mouse adenomas. Gastroenterology, 111: 1134–1140, 1996.

45. Subbaramaiah, K., Telang, N., Ramonetti, J. T., Araki, R., DeVito, B., Weksler, B. B., and Dannenberg, A. J. Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res., 56: 4424–4429.

46. Lanza, F. L. A review of gastric ulcer and gastroduodenal injury in normal volunteers receiving aspirin and other non-steroidal anti-inflammatory drugs. Scand. J. Gastroenterol., 24: 24–31, 1989.

47. Cay, Y., Appelkvist, E-L., and DePierre, M. Hepatic oxidatives stress and related deferences during treatment of mice with acetylsalicylic acid and other peroxisome proliferators. J. Biochem. Toxicol., 10: 87–94, 1995.

48. Hixson, L. J., Alberts, D. S., Krutzsch, M., Einsphar, J., Brendel, K., Gross, P. H., Paranka, N. S., Baier, M., Emerson, S., Pamukcu, R., and Burt, R. W. Antiproliferative effect of nonsteroidal anti-inflammatory drugs against human colon cancer cells. Cancer Epidemiol. Biomark. Prev., 3: 433–438, 1994.

49. Miyamoto, T., Ogino, N., Yamamoto, S., and Hayaishi, O. Purification of prostaglandin endoperoxide synthase from bovine vesicular gland microsomes. J. Biol. Chem., 251: 2629–2636, 1976.

50. Mizuno, K., Yamamoto, S., and Lands, W. E. M. Effects of non-steroidal anti-inflammatory drugs on fatty acid cyclooxygenase and prostaglandin hydroperoxidase activities. Prostaglandins, 23: 743–757, 1982.

51. Yao, R., Rioux, N., Castonguay, A., and You, M. Inhibition of COX-2 and induction of apoptosis: two determinants of NSAIDs chemopreventive efficacies in mouse lung tumorigenesis. Proc. Am. Assoc. Cancer Res., 39: 195, 1998.

52. Martin, U., and Prescott, L. F. The interaction of paracetamol with frusamide. Br. J. Clin. Pharmacol., 37: 464–467, 1994.

53. Clissold, S. P. Paracetamol and phenacetin. Drugs, 32: 46–59, 1986.

54. Shen, W., Kamendulis, L. M., Ray, S. D., and Corcoran, G. B. Acetaminophen-induced cytotoxicity in cultured mouse hepatocytes: correlation of nuclear Ca^{2+} accumulation and early DNA fragmentation with cell death. Toxicol. Appl. Pharmacol., 111: 242–254, 1991.