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Inhibitors of lipoxygenase: a new class of cancer chemopreventive agents

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5-Lipoxygenase is a key enzyme in the metabolism of arachidonic acid to leukotrienes. The preventive efficacy of 5-lipoxygenase inhibitors against lung tumorigenesis was determined in A/J mice given the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in drinking water from week 0 to week +7. Groups of 25 mice were fed either: acetylsalicylic acid (ASA), a cyclooxygenase inhibitor; A-79175, a 5-lipoxygenase inhibitor; MK-886, an inhibitor of the 5-lipoxygenase activating-protein; a combination of ASA and A-79175 from weeks −2 to +23. ASA, A-79175 and MK-886 reduced lung tumor multiplicity by 44, 75 and 52% respectively. Furthermore, A-79175 reduced tumor incidence by 20%. Administration of A-79175 and MK-886 decreased the mean tumor volume by 64 and 44% respectively. Lung tumor multiplicity was directly proportional to tumor volume. The combination of ASA and A-79715 was the most effective preventive intervention and reduced lung tumor multiplicity by 87% and lung tumor incidence by 24%, demonstrating that inhibition of both 5-lipoxygenase and cyclooxygenase is more effective than inhibition of either pathway alone. NNK treatment increased plasma prostaglandin E_2 levels from 49 to 260 pg/ml and plasma LTB_4 levels from 29 to 71 pg/ml. Incubation of 82-132 and LM2 murine lung tumor cells with MK-886 and A-79715 decreased cell proliferation in a concentration-dependent manner. Soybean lipoxygenases with or without murine lung microsomal proteins metabolized NNK by α-carbon hydroxylation (9.5% of the metabolites) and *N*-oxidation (3.9%). Activation of NNK by α-carbon hydroxylation was inhibited by addition of arachidonic acid and A-79715. Possible mechanisms of action of 5-lipoxygenase inhibitors include inhibition of tumor growth and lipoxygenase-mediated activation of NNK. These studies suggest that inhibitors of 5-lipoxygenase may have benefits as preventive agents of lung tumorigenesis.

Introduction

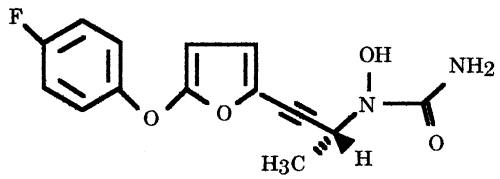
Arachidonic acid, released from membrane phospholipids upon cell stimulation, is converted to leukotrienes by lipoxygenases or to prostanoids by cyclooxygenases. Inhibition of cyclooxygenases delays tumorigenesis in animals and humans (1,2). Our laboratory was the first to demonstrate that non-steroidal anti-inflammatory drugs (NSAIDs*), such as acetylsalicylic acid (ASA) and sulindac, inhibit cyclooxygenases activity and chemically induced tumorigenesis in the lungs of A/J mice (3–5). In this animal model the highest level of inhibition (60%) was observed with ASA (5). The cumulative efficacy of NSAIDs combined with other prophylactic agents has not been investigated.

5-Lipoxygenase (5-LO) catalyzes both the oxygenation of arachidonic acid to (5*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid and its subsequent conversion to leukotriene A_4, the precursor of leukotriene B_4 (LTB_4) (6). The synthesis of leukotrienes also requires the membrane bound 5-lipoxygenase activating protein (FLAP) (7). FLAP activates 5-LO by specifically binding arachidonic acid and by transferring this substrate to the enzyme (8). Leukotrienes are potent mediators of numerous biological processes, including chemotaxis, vascular permeability and smooth muscle contraction (9). Avis *et al.* reported that mRNA for 5-LO and FLAP are expressed in lung tumor cell lines (10). Lipoxygenase products have been shown to stimulate cellular proliferation either directly or as intermediates in the mitogenic signal pathway mediated by growth factors (11,12). The 5-LO substrate arachidonic acid limits the growth rate of hepatoma 7288CTC cells in rats (13). 5-LO activity was significantly higher in colon tumors than in normal mucosa of azoxymethane-treated rats (14). Recently, Hussey and Tisdale observed that the lipoxygenase inhibitors BWA4C, BWA70C and zileuton inhibited proliferation of murine colon adenocarcinoma cell lines and transplanted tumors (15). These results suggest that 5-LO activity could be used as a biomarker of tumorigenesis. The efficacy of 5-LO inhibitors against lung cancer has never been investigated and the mechanism(s) by which lipoxygenase inhibitors control tumor growth remain(s) unknown.

N-hydroxyureas are among the most potent orally active 5-LO inhibitors. The most active of these, *R*(+)-*N*-[3-[5-(4-fluorophenyl)-2-furauyl]-1-methyl-2-propynyl]-*N*-hydroxyurea (A-79175; Figure 1), is a potent inhibitor of LTB_4 formation in rats and humans (16). Alternatively, leukotriene biosynthesis can be inhibited by compounds that bind to FLAP, such as 3-[1-(4-chlorobenzyl)-3-t-butyl-thio-t-isopropyl-indol-2-yl]-2,2-dimethylpropanoic acid (MK-886, formely L-663,536; Figure 1; 17). However, the efficacy of these two compounds in preventing carcinogenesis has never been investigated. We have hypothesized that A-79175 and MK-886 could effectively inhibit lung tumorigenesis in A/J mice.

The potent tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces lung tumors in rodents (18). NNK is present in cigarette smoke and most likely involved in lung tumorigenesis in smokers (18,19). Metabolic activation of NNK by cytochrome P450s is well documented (20). Oxygen radicals also play a role in the mediation of DNA damage by NNK (21). Smith *et al.* suggested

***Abbreviations:** A-79175, *R*(+)-*N*-[3-[5-(4-fluorophenyl)-2-furauyl]-1-methyl-2-propynyl]-*N*-hydroxyurea; ASA, acetylsalicylic acid; FLAP, 5-lipoxygenase activating protein; 5(S)HETE, 5(*S*)-hydrooxyeicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid; 5-LO, 5-lipoxygenase; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NSAIDs, non-steroidal anti-inflammatory drugs; MK-886, 3-[1-(4-chloro-benzyl)-3-t-butyl-thio-t-isopropyl-indol-2-yl]-2,2-dimethylpropanoic acid (formerly L-663,536); MTT, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide; PGE_2, prostaglandin E_2.



A-79175

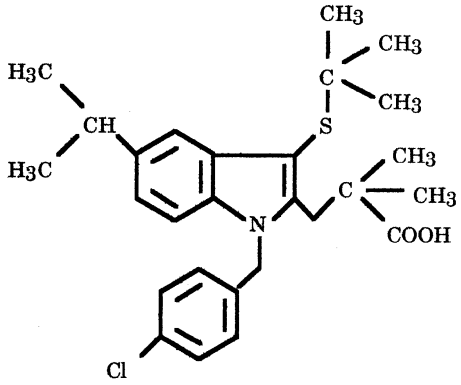


Fig. 1. Chemical structure of A-79175 and MK-886.

that lipoxygenases play a role in the oxidation of NNK in human lung microsomes (22). They observed higher levels of NNK metabolites following co-incubation with microsomal proteins and soybean lipoxygenases. The role of lipoxygenases in NNK metabolic activation remains unclear.

The aims of this study were: (i) to evaluate the efficacy of 5-LO and FLAP inhibitors to prevent lung tumorigenesis in A/J mice, alone or in combination with a cyclooxygenase inhibitor such as ASA; (ii) to investigate the effect of 5-LO or FLAP inhibitors on murine lung cell proliferation; and (iii) to determine if NNK is bioactivated by lipoxygenases.

Materials and methods

Chemicals

NNK (99% pure by thin layer chromatography) and [5-^{3}H]NNK (99% pure as determined by HPLC) were purchased from Chemsyn Science Laboratories (Lenexa, KS). The synthesis of metabolites used as standards in HPLC analysis of NNK metabolism has been reported (23). ASA (99.5% pure), bovine serum albumin, dimethylsulfoxide, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, hydrochlorothiazide, indomethacin, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT), NADP$^+$, soybean lipoxygenases and sulindac were purchased from Sigma (St Louis, MO). A-79175 (98% pure by HPLC) was a gift from Abbott Laboratories (Abbott Park, IL). MK-886 (99% pure by HPLC) was a gift from Merck Frosst (Pointe-Claire, Canada). Triethylacetate buffer and acetohydroxamic acid were purchased from Fluka Chemika-BioChemika (Ronkonkoma, NY). The standard [5,6,8,11,12,14,15-^{3}H(N)]prostaglandin E_2 (PGE_2) (200 Ci/mmol) was supplied by NEN DuPont (Mississauga, Canada). Arachidonic acid, LTB_4, leukotriene C_4 (LTC_4) and 5(*S*)-hydrooxyeicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid [5(S)HETE] were purchased from Cayman Chemicals (Ann Arbor, MI). Dulbecco's modified Eagle's medium, minimal essential medium, fetal bovine serum and penicillin/streptomycin solution were from Life Technologies (Burlington, Canada).

Lung tumor assay in A/J mice

Seven-week-old A/J female mice (18–22 g) were obtained from Jackson Laboratories (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 cycle). Animals were treated in accordance with approved institutional protocols and following the 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 1 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 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 five mice from each cage were weighed weekly.

Details of treatments with NNK and chemopreventive agents are included in Table I. Group 1 (22 mice) received the diet without chemopreventive agents and was given tap water *ad libitum*. Groups 2–6 (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 the chemopreventive agents were given to groups 3–6, starting 2 weeks before the treatment with NNK and continuing throughout the assay (weeks –2 to +23). Chemopreventive agents were given at non-toxic doses. On a weight basis, the concentration of MK-886 given to the mice was below the 500 mg doses administered and well tolerated by human (24). The doses of ASA was <50% of the maximum tolerated dose.

Sixteen weeks post-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. Representative lung samples were embedded in paraffin and stained for histopathological examination. Tumors were identified as lung adenomas. Stomachs were fixed *in situ* with 0.5 ml 10% formalin, excised and stored in formalin. Papillomas >1 mm were counted.

Analysis of drug stability in diets

ASA. Diet samples containing 294 mg ASA/kg diet were recovered from feeders after a 4 day period of feeding. Two gram samples of diet were extracted with 20 ml of a 9:1 mixture of diethyl ether:0.01 N HCl. A 100 μl aliquot of hydrochlorothiazide solution (100 μg/ml methanol) was added as internal standard to 2 ml aliquots of supernatant. Samples were evaporated to dryness and resuspended in 200 μl 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 consisted of 95% methanol:0.02 M potassium phosphate buffer, pH 5.0 (40:60 v/v). Elution at a flow rate of 1.0 ml/min was monitored at 254 nm. Hydrochlorothiazide eluted at 3 and ASA 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.

A-79175. Stability of A-79175 was determined as described for ASA with the following modifications: 100 μl of indomethacin (200 μg/ml) were added as internal standard and the HPLC mobile phase consisted of acetonitrile:10 mM acetohydroxamic acid in 8 mM triethylamine acetate buffer, pH 6.5 (55:45 v/v). Indomethacin eluted at 2.5 and A-79175 at 4 min (16).

MK-886. Stability of MK-886 was evaluated as described for ASA with the following modifications: 100 μg/ml sulindac were added as internal standard and the HPLC mobile phase consisted of methanol:0.01 M potassium phosphate buffer, pH 7.0. A gradient of 60–95% methanol was used over a 50 min period. Sulindac and MK-886 were eluted at 3 and 25 min respectively. Elution at a flow rate of 1 ml/min was monitored at 290 nm.

Determination of plasma A-79715 and MK-886 levels

Plasma levels of A-79175 and MK-886 were measured in five mice from groups 4 and 6 of the lung tumor assay (Table I). Proteins were removed from plasma by methanol precipitation: 200 μl HPLC grade methanol were added to 100 μl plasma and centrifuged at 10 000 *g* at 25°C for 5 min. This sequence was repeated once and the pooled supernatants were filtered through a 0.45 μm Millex-LCR cartridge filter (Millipore, Bedford, MA). Indomethacin or sulindac was added as internal standard for determination of A-79175 and MK-886 levels respectively and 25 μl supernatant were used for HPLC as described above.

Assay of plasma PGE_2 and LTB_4

PGE_2 was assayed in the plasma of mice from the lung tumor bioassay, 16 weeks post-NNK treatment. At the time of sacrifice, blood was collected by

Table I. Effects of chemopreventive agents on lung and stomach tumorigenesis in A/J mice.

Group no.	NNK (mg/mouse)[a] (mean ± SD)	Chemopreventive agent[b]	Dose of agent (mg/kg diet) (mmol/kg body wt/day)	Lung tumors			Stomach tumors
				Multiplicity (mean ± SE)[c,d]	Incidence[e]	Volume (mm^3) (mean ± SE)[f]	Incidence[g]
1 (negative)	None	None	None	0.32 ± 0.12	6/22*	None	0/22
2 (positive)	9.09 ± 0.02	None	None	8.65 ± 1.21	24/24	2.24 ± 0.25	3/24
3	9.10 ± 0.05	ASA	294 (2.45)	4.83 ± 0.52*	24/24	1.43 ± 0.14	1/24
4	9.09 ± 0.04	A-79175	75 (0.37)	2.16 ± 0.33**	20/25*	0.79 ± 0.07	1/25
5	9.06 ± 0.04	ASA + A-79175	294 + 75 (2.45 + 0.37)	1.12 ± 0.22**[§]	19/25*	1.03 ± 0.13	1/25
6	9.09 ± 0.05	MK-886	25 (0.08)	4.16 ± 0.38**	25/25	1.25 ± 0.16	2/25

[a]NNK was given in drinking water from week 0 to +7. Total dose was estimated to be 2.2 mmol/kg body wt.
[b]Feeding the chemopreventive agents was started on week –2 and pursued to the end of the assay (week +23).
[c]Statistically different from positive control, one factor ANOVA: *$P < 0.05$, **$P < 0.001$. Statistically different from group 4, one factor ANOVA: [§]$P < 0.01$.
[d]Groups 2–6 are statistically different from negative control (group 1) ($P < 0.0005$).
[e]Statistically different from negative control (group 2), χ^2: *$P < 0.001$.
[f]Determined from 23–96 tumors located in 14–25 mice/group, statistically different from group 2, one factor ANOVA: $P < 0.005$.
[g]Tumor-bearing mice had 1 or 2 tumor(s)/mouse.

cardiac puncture in heparinized microtainer tubes and kept on ice. The blood was centrifuged at 1500 *g* at 4°C for 5 min and the resulting plasma stored at –70°C until analysis. Samples showing hemolysis were discarded. PGE_2 was extracted as described by Powell (25). Briefly, a 10 µl aliquot of [5,6,8,11,12,14,15-^{3}H(N)]PGE_2 (20 000 d.p.m.) tracer was added to each sample to determine recovery. Each sample of plasma (200 µl) was mixed with 2.0 ml methanol, incubated at 4°C for 5 min and then centrifuged at 1500 *g* for 10 min to remove insoluble proteins. Pellets were washed with 2.0 ml methanol and the supernatants kept on ice. The combined supernatants were diluted with four volumes of 0.1 M phosphate buffer (pH 4.0). A Sep-Pak Plus C-18 reverse phase cartridge (Waters Associates) was rinsed once with 5 ml ultra-pure water and once with 5 ml HPLC grade hexane. Samples were loaded in the cartridge and PGE_2 was eluted with 5 ml ethylacetate containing 1% methanol. After evaporating the samples to dryness, the residue was dissolved in 0.5 ml immunoassay buffer. Two aliquots of 250 µl were used to measure ^{3}H and to determine PGE_2 with a competitive monoclonal enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI) according to Pradelles *et al.* (27).

LTB_4 was extracted as in the PGE_2 assay with the following modifications: LTB_4 was eluted from the C-18 cartridge with 5 ml ethanol:water (90:10 v/v) and the samples resuspended in 150 µl EIA buffer after evaporation (25,26). Recovery was determined by extraction of a known amount of LTB_4 standard. The plasma levels of LTB_4 were measured by a competitive LTB_4 monoclonal enzyme immunoassay (Cayman Chemicals).

Preparation of microsomes

Lung microsomes from A/J female mice were prepared by differential centrifugation as described previously (28). Protein content as determined by the method of Lowry *et al.* using bovine serum albumin as the standard was 1.95 mg protein/ml microsomes (29).

Assay of NNK metabolism by P450s and lipoxygenases

Microsomal incubations were performed as described by Smith *et al.* (30) with some minor modifications. The incubation mixture consisted of 5 mM glucose 6-phosphate, 1.52 U glucose 6-phosphate dehydrogenase, 1 mM $NADP^+$, 1 mM EDTA, 3 mM $MgCl_2$, 10 µM (13 µCi) [5-^{3}H]NNK and 100 mM sodium phosphate buffer (pH 7.4) in a total volume of 800 µl. Following incubation at 37°C for 10 min, the reaction was initiated by addition of 0.5 mg lung microsomal proteins. The reaction was incubated for 30 min and stopped by addition of 200 µl 25% zinc sulfate and 200 µl saturated barium hydroxide. Some incubations were carried out with soybean lipoxygenases (10 000 U) with or without arachidonic acid (100 µM) (Table III). Incubations with 100 µM A-79715 were also performed (incubation 9). Incubation 10 was done to exclude inhibition of P450s by A-79175 as a mechanism of inhibition of tumorigenesis by this agent. Incubation 8 was performed with lipoxygenase co-factor under the following conditions: 8 mM EDTA, 2 mM $CaCl_2$, 10 µM (13 µCi) [5-^{3}H]NNK, 10 000 U lipoxygenases and water in a total volume of 800 µl. Precipitated samples were centrifuged at 14 000 *g* for 30 min and filtered through a 0.45 µm Millex-LCR cartridge filter (Millipore). Five hundred microliters of the filtrate and 7 µl metabolite standards were co-injected onto a reverse phase HPLC system, using a Spherisorb ODS2 5 µm column (Jones Chromatography, Columbus, OH). NNK and its metabolites were eluted with sodium acetate buffer (pH 6.0) and methanol as described previously (31). The elution was monitored at 254 nm and a 1 ml fraction was collected. Five milliliters of Scintisafe Plus (Fisher Scientific, Montreal, Canada) were added to each fraction and radioactivity was measured by liquid scintillation spectroscopy. Recovery of total radioactivity during the HPLC analysis was >65%.

Cell lines

Murine lung cells, 82-132, originating from a type II solid carcinoma, were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin/streptomycin (32). The LM2 murine lung epithelial cells, derived from a papillary tumor, were cultured in minimum essential medium alpha 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 chemopreventive agents. Chemopreventive agents were dissolved in dimethylsulfoxide. The concentration of dimethylsulfoxide in all wells, including control wells, was 2%. After 72 h incubation at 37°C, 25 µl of a sterile solution of MTT in sterile phosphate-buffered saline (5 mg/ml) were added to each well and incubation was continued for 2 h at 37°C. Cells were lysed with 100 µl extraction buffer, which consists of 20% w/v SDS dissolved in a 50% solution of *N′,N′*-dimethylformamide in water, pH 4.7. Optical densities at 570 nm were measured after 18 h incubation at 37°C (33). Assays with lipoxygenase metabolites were performed to determine if those metabolites are implicated in cell proliferation (Table IV). 5(S)HETE, LTC_4 and LTB_4 in ethanol solution were added to the cells at a concentration of 7.5 µM in the presence or absence of 20 µM A-79175.

Statistical analysis

Multiplicity and volumes of lung adenomas, PGE_2 and LTB_4 levels and NNK metabolism were compared using one factor ANOVA. Incidence of lung adenomas were compared by χ^2 analysis. Percentages of cellular proliferation were compared by two-sided Student's *t*-tests. Differences between samples were considered statistically significant at $P < 0.05$.

Results

Lung tumor assay

NNK given at a cumulative dose of 9.06–9.11 mg/mouse, for 7 weeks, prevented normal body weight gain (Figure 2). Body weights of NNK-treated mice sharply increased after cessation of NNK treatment, but never reached those of untreated mice. Body weights of mice treated with NNK + chemopreventive agents were not statistically different from untreated mice at the time of sacrifice. Chemopreventive agents were given at non-toxic doses. Food consumption of mice treated with NNK + chemopreventive agents was not statistically different from consumption by untreated mice (range 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.

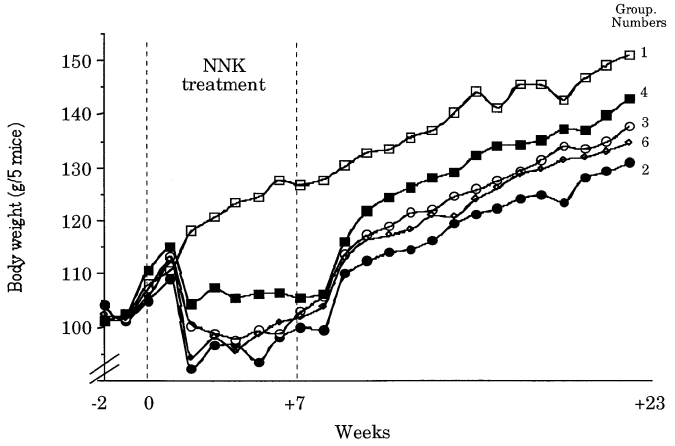


Fig. 2. Body weights of A/J mice during lung tumor assay. Each point corresponds to the mean of five cages. SD were <10%. Group numbers correspond to the numbers in Table I: 1, negative control; 2, NNK only; 3, ASA + NNK; 4, A-79175 + NNK; 6, MK886 + NNK. NNK was given from week 0 to +7 and the chemopreventive agents were given from week −2 to +23. Groups 2–4 and 6 were statistically different from group 1 at the end of the NNK treatment (week +7) (one factor ANOVA, $P < 0.05$). This difference in body weight was smaller after the end of the NNK treatment and not statistically different at week +23 (one factor ANOVA).

Screening of the mouse colony indicated no viral or bacterial infection at the end of the lung bioassay. One mouse from group 2 and one mouse from group 3 were sacrificed before the end of the assay due to diarrhea and dehydration and were eliminated from the study.

The effects of chemopreventive agent treatment on lung tumorigenesis are shown in Table I. As expected, untreated mice (group 1) had a small number of spontaneous tumors (0.32 ± 0.12 per mouse). Treatment with NNK (group 2) induced a mean of 8.65 tumors/mouse. Feeding ASA throughout the study reduced lung tumor multiplicity by 44%. A-79175 treatment reduced lung tumor multiplicity by 75% and incidence by 20%. The combination of ASA and A-79175 was more effective than the treatment with either one of the two chemopreventive agents, reducing lung tumor multiplicity by 87% ($P < 0.01$), but the tumor incidence remained similar to the A-79175-treated group. MK-886 decreased tumor multiplicity by 52%.

Volumes of lung tumors in NNK-treated mice averaged 2.24 ± 0.25 mm^3. ASA treatment reduced tumor volumes by 35% ($P < 0.05$), while MK-886 and A-79715 decreased NNK-induced tumor volumes by 44 and 65% respectively ($P < 0.05$). A linear correlation was observed between inhibition of lung tumor multiplicity and tumor volume ($r^2 = 0.91$; data not shown). The incidence of gastric papillomas was low and no statistical difference was observed between the groups (Table I).

The chemopreventive agents mixed with AIN-76A diet were relatively stable under the conditions of feeding. We recovered 92 and 97% of A-79175 and MK-886 from the diets under feeding conditions. In the case of ASA, 93% of the drug was recovered from the diet, but a significant variation (up to 20%) was observed between samples (data not shown).

Determination of A-79175 and MK-886 plasma level

We measured the A-79175 and MK-886 plasma levels in five mice from the lung tumor bioassay (groups 4 and 6 respectively, Table I). The mean plasma level, after a dietary treatment of 25 weeks, was 13.5 ± 2.4 μM in A-79175-treated mice ($n = 5$), while the plasma MK-886 level was 37.1 ± 4.0 μM ($n = 5$) (data not shown). Accordingly, the effects of chemopreventive agents on tumor cell proliferation were investigated from 0 to 40 μM.

Table II. Plasma PGE_2 and LTB_4 levels in A/J mice after NNK treatment with or without chemopreventive agents

Group[a]	Carcinogen	Chemopreventive agent	PGE_2 (pg/ml)[b] (mean ± SE)	LTB_4 (pg/ml)[b] (mean ± SE)
1	None	None	49 ± 4 (8)	29 ± 8 (4)
2	NNK	None	260 ± 68* (17)	71 ± 8** (5)
3	NNK	ASA	120 ± 35 (6)	n.d.
4	NNK	A-79175	254 ± 39** (6)	27 ± 4† (5)
5	NNK	A-79175 + ASA	96 ± 18 (6)	n.d.
6	NNK	MK-886	219 ± 81 (8)	23 ± 4 (5)

[a]Groups are identical to those in Table I.
[b]Number of determinations is equal to number of mice, as indicated in parentheses. Statistically different from group 1, one factor ANOVA, *$P < 0.05$, ** $P < 0.005$. Statistically different from group 2, one factor ANOVA, $P < 0.05$.
n.d., not determined.

Inhibition of PGE_2 and LTB_4 synthesis

Plasma PGE_2 level in untreated mice was 49 ± 4 pg/ml (Table II). NNK treatment raised this level to 260 ± 68 pg/ml ($P < 0.05$). As expected, A-79175 and MK-886 administration had no effect on plasma PGE_2. ASA reduced the NNK-induced PGE_2 plasma levels to 120 ± 35 pg/ml, a concentration similar to that measured in ASA + A-79715-treated mice. More than 85% of PGE_2 and LTB_4 were extracted from plasma. Plasma LTB_4 level in untreated mice was 29 ± 8 pg/ml (Table II). NNK treatment raised this level to 71 ± 8 pg/ml ($P < 0.005$). A-79175 or MK-886 treatment of NNK-exposed mice lowered plasma LTB_4 back to untreated mouse levels.

Metabolism of NNK by microsomes and lipoxygenases

NNK is bioactivated by hydroxylation of the carbons adjacent to the *N*-nitroso nitrogen, producing hydroxy acid, keto acid, diol and keto alcohol. NNK *N*-oxide and 4-methylnitrosamino-1-(3-pyridyl)-1-butanol *N*-oxide result from NNK pyridine *N*-oxidation (20). A/J mouse lung microsomes metabolized 10 μM [5-^{3}H]NNK by α-carbon hydroxylation (9.5%) and *N*-oxidation (3.9%) (incubation 1, Table III). Addition of 10 000 U soybean lipoxygenases to microsomal proteins raised the amount of NNK metabolized to 21% ($P < 0.05$) (incubation 2). Elimination of co-factors (incubation 4) decreased total metabolism from 13.5 to 5.8%, but addition of arachidonic acid did not increase NNK metabolism (incubation 3). In the absence of microsomal proteins lipoxygenases metabolized 12% of the NNK (incubation 7). Arachidonic acid added to lipoxygenases inhibited NNK bioactivation by 24% (incubation 8). Replacement of the NADPH generating system by ATP, the usual co-factor of lipoxygenases (incubation 9), did not affect the amount of NNK metabolites. As expected, addition of A-79715 to lipoxygenases inhibited NNK α-carbon hydroxylation by 66% ($P < 0.05$) and *N*-oxidation by 33% (incubation 10). This inhibition by A-79175 seems totaly related to lipoxygenase activity, since incubation of microsomal protein with this agent did not affect NNK bioactivation (incubation 4).

Inhibition of cell proliferation by chemopreventive agents

The effects of MK-886 and A-79175 on proliferation of both 82-132 and LM2 cell lines are shown in Figure 3. In general, MK-886 and A-79175 were more effective antiproliferative agents than ASA. The sensitivity of the two cell lines to

Table III. Metabolism of NNK by mouse lung microsomes and lipoxygenases

Incubation variables					NNK metabolites		
Incubation	Microsomes (mg protein)	Lipoxygenases (inhibitor)	Arachidonic acid	Co-factor	α-Carbon hydroxylation (%)[a]	*N*-oxidation (%)[b]	NNK + NNAL (%)[cd]
1	0.5	None	None	NADPH	9.5 ± 0.4	3.9 ± 1.3	86.5 ± 1.7
2	0.5	10 000 U	None	NADPH	11.3 ± 0.7	9.8 ± 1.5*	78.9 ± 1.0*
3	0.5	10 000 U	100 μM	NADPH	9.3 ± 0.9	3.8 ± 0.5	86.8 ± 0.5
4	0.5	None (A-79175)	None	NADPH	8.1 ± 0.5	5.9 ± 0.3	86.0 ± 0.7
5	0.5	None	None	None	4.0	1.8	94.2
6	0.5	None	100 μM	None	3.8	1.2	95.1
7	None	10 000 U	None	NADPH	9.4 ± 1.2	3.4 ± 0.4	87.2 ± 0.9
8	None	10 000 U	100 μM	NADPH	6.8 ± 0.3**	2.9 ± 0.3**	90.3 ± 0.5**
9	None	10 000 U	None	ATP	10.0 ± 1.7	2.4 ± 0.3	87.1 ± 0.9
10	None	10 000 U (A-79175)	None	NADPH	4.2 ± 0.7**	2.3 ± 0.5	93.5 ± 0.4**

[a]The percentage of metabolites of NNK and NNAL. Mean of three determinations ± SD.
[b]Statistically different from group 1, one factor ANOVA (repeated measures): *$P < 0.01$.
[c]Statistically different from group 7, one factor ANOVA (repeated measures): **$P < 0.05$.
[d]Percentage of initial amount of NNK
The metabolic pathway of NNK has been detailed by Jorquera *et al.* (28).

the chemopreventive agents was slightly different. Significant growth inhibition of cell line 82-132 occurred with an EC_{50} of 4.0 μM for MK-886, 30 μM for A-79175 and 140 μM for ASA. In the case of cell line LM2, the EC_{50} values were 10 μM for both MK-886 and A-79715 and >200 μM for ASA. MK-886 totally inhibited 82-132 and LM2 proliferation, while A-79175 brings only LM2 growth to 0%. As shown in Table IV, 5(S)HETE increased growth of cell line 82-132 by 16% ($P < 0.05$), both in the presence and absence of A-79175. LTB_4 and LTC_4 had no effect on cell proliferation, with or without A-79715 exposure.

Discussion

Arachidonic acid is metabolized to prostaglandins and thromboxanes by cyclooxygenases or to leukotrienes by lipoxygenases. In contrast to inhibitors of cyclooxygenases, such as ASA, an established chemopreventive agent, the efficacy of lipoxygenase inhibitors in the prevention of tumorigenesis has received little attention (2). In this study we observed that inhibitors of 5-LO reduced lung tumorigenesis in A/J mice. Inhibition of 5-LO was also associated with inhibition of murine lung tumor cell proliferation. We showed that lipoxygenases metabolize the tobacco-specific nitrosamine NNK and that A-79175 inhibits NNK activation by lipoxygenases. Results of this study suggest that 5-LO inhibitors are efficient preventive agents of lung tumorigenesis. Possible mechanisms of action could involve inhibition of cell proliferation as well as inhibition of lipoxygenases-mediated NNK activation.

The first aim of this study was to evaluate the efficacy of 5-LO inhibitors in the prevention of lung tumorigenesis in A/J mice. Mice were exposed to low doses of NNK for an extended period of time to mimic the exposure of smokers to this carcinogen. Tobacco smoke is immunosuppressive in smokers and sustained exposure to NNK is immunosuppressive in A/J mice (34,35). As such, the A/J mouse is particularly suitable for testing NSAIDs as cancer preventive agents (36). In this study the doses of A-79715 and MK-886 were selected according to their inhibitory ED_{50} for leukotriene synthesis in rats (16,37). Inhibitors of 5-LO are currently being developed for treatment of asthma. Asthmatic patients show sustained improvements after a 6 month daily administration of zileuton, a 5-LO inhibitor, without signs of significant side-effects (38). Furthermore, asthmatic patients are known to be at higher risk for lung cancer than are healthy individuals (39–41). This provides a rationale for investigating possible reduced risk of lung cancer among patients as a result of 5-LO inhibitor use. We are the first to report the preventive efficacy of 5-LO inhibitors against lung cancer. The 5-LO specific inhibitor A-79175 reduced lung tumor multiplicity and incidence. We also observed an inhibition of lung tumor multiplicity with MK-886, a FLAP inhibitor. The chemopreventive efficacy of leukotriene synthesis inhibitors does not seem to be limited to lung tumorigenesis; Jiang *et al.* have inhibited benzo[*a*]pyrene-induced skin tumorigenesis in CD-1 mice with the 5-LO inhibitor TMK688 (42). Thus, inhibitors of leukotriene synthesis appear to be effective chemopreventive agents in multiple tissues.

In this study lung tumor multiplicity was reduced in mice fed ASA, an inhibitor of the cyclooxygenase pathway of arachidonic acid metabolism. The adverse effects of NSAIDs are significant and could drastically limit their development as chemopreventive agents. The major adverse effect is gastrointestinal toxicity, which ranges from mild dyspepsia to bleeding and perforation, leading to death in some cases (43). NSAIDs are also known to induce several nephrological syndromes, including acute renal failure, chronic renal injury, abnormalities of water metabolism and perturbations in sodium and potassium homeostasis (44). The preventive efficacy of NSAIDs, like sulindac and ASA, shows a logarithmic dependence with respect to the dosage administered, while toxic effects increase with respect to dose (3,35,45). The use of lower doses of NSAIDs in combination with other cancer preventive agents warrants investigation. As shown in Table I, a combination of ASA and A-79175 was more potent than either agent alone. Thus, inhibition of both pathways of arachidonic acid metabolism (cyclooxygenases and 5-LO) enhances lung tumor prevention. We are currently exploring the potential of cyclooxygenase-2 specific inhibitors as preventive agents (N.Rioux and A.Castonguay, submitted for publication).

The lung-specific carcinogen NNK induced both prostaglandin and leukotriene synthesis in A/J mice. Mice bearing NNK-induced tumors had a PGE_2 plasma level 5-fold higher

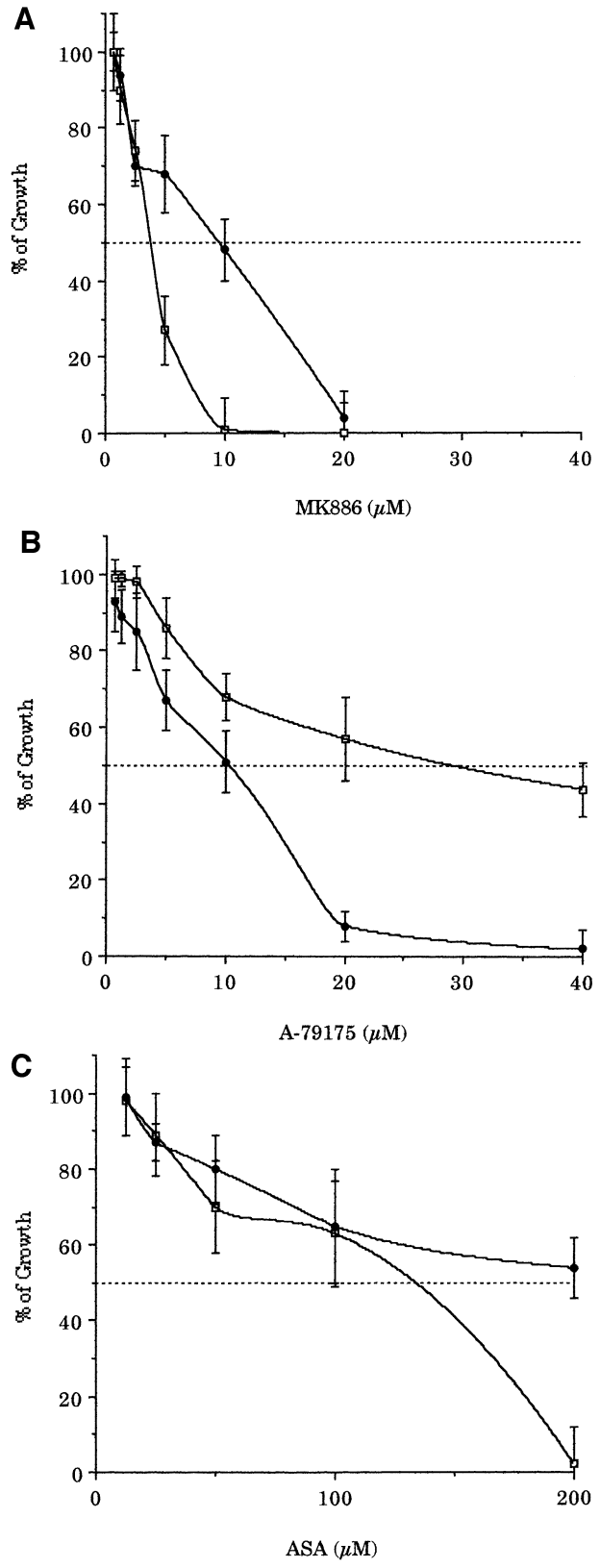


Fig. 3. Inhibition of murine lung cell proliferation by various chemopreventive agents. Each point corresponds to the mean of three determinations ± SE. (**A**) MK886; (**B**) A-79175; (**C**) ASA. □, 82-132 cell line; ●, LM2 cell line. The 100% growth value corresponds to growth of untreated cells.

Table IV. Effects of lipoxygenase products on 82-132 cell growth

Lipoxygenase metabolite	Growth (%)	
	− A-79175 (mean ± SD)	+ A-79175 (mean ± SD)
None	100	69 ± 5
5(S)HETE	116 ± 10*	85 ± 11[a]
LTC_4	102 ± 7	71 ± 4
LTB_4	100 ± 8	68 ± 6

[a]Statistically different from the control, Student's *t*-test, $P < 0.05$.
Mean ± SD of three determinations for LTC_4 and LTB_4; five determinations for 5(S)HETE. A-79175 was added at a concentration of 20 μM. 5(S)HETE, LTC_4 and LTB_4 were added at a concentration of 7.5 μM.

than untreated animals (Table II). We previously reported that PGE_2 level was doubled immediately following NNK treatment of mice and before macroscopic detection of any tumor (35). Similarly, Rao *et al.* observed an elevation of cyclooxygenase and lipoxygenase metabolites in colorectal tissues of rats bearing azoxymethane-induced tumors (14). The higher level of PGE_2 observed in mice bearing NNK-induced tumors could reflect induction of cyclooxygenase-2. As expected, we observed in this study that ASA partially inhibited NNK-induced PGE_2 synthesis. Induction of 5-LO by a carcinogen has never been reported. In this study plasma LTB_4 concentrations more than doubled in mice bearing NNK-induced tumors. A-79175 and MK-886 returned elevated plasma LTB_4 levels in NNK-treated mice to those of untreated mice and, as expected, did not affect PGE_2 levels (Table II). Furthermore, MK-886 treatment did not inhibit PGE_2 levels in guinea pigs (37). However, A-79175 was a more effective inhibitor of lung tumorigenesis than MK-886, despite both drugs inhibiting LTB_4 synthesis to similar extents. We conclude that LTB_4 concentration in mice reflects qualitatively but not quantitatively the preventive efficacy of the agents.

Our results suggest that prevention of lung tumorigenesis by 5-LO inhibitors involves inhibition of cell proliferation. In the A/J mouse lung tumor assay tumor growth beyond a minimal volume is essential to reach a macroscopically detectable mass 16 weeks after carcinogen treatment. As shown in Figure 2, growth of murine lung cells derived from a type II solid lung carcinoma (82-132) and from lung epithelial cells (LM2) was inhibited by A-79715 and MK-886. The effective concentrations of the agents used for the lung tumor bioassay were achieved in plasma of mice fed the two agents. A-79175 and MK-886 were ~10 times more potent inhibitors of proliferation than ASA. Our results parallel those of Avis *et al.*, who observed selective inhibition of human lung cancer cell growth *in vitro* by 5-LO inhibitors such as nordihydroguaiaretic acid, AA861 and MK-886 (10). Two other lipoxygenase inhibitors, esculetin and NDGA, have been shown to suppress proliferation of breast cancer cells *in vitro* (46). As shown in Table IV, the addition of 5(S)HETE partially prevented the growth inhibition due to 5-LO inhibitors, confirming the implied role of this 5-LO metabolite in cell growth regulation. Quite unexpectedly, LTB_4 and LTC_4 had no effect on cell proliferation, showing the complexity and the diverse functions of 5-LO metabolites. In this study the preventive efficacy of the agents positively correlates with the mean lung tumor volume, suggesting that they possess an *in vivo* antiproliferative effect.

Considering that the inhibitors of 5-LO were given during the NNK treatment, it was important to determine how the inhibitors could affect NNK activation. While pulmonary

cytochrome P450s are clearly implicated in activation of NNK by α-carbon hydroxylation, Smith *et al*. concluded that lipoxygenases were also involved (20,22). These authors suggested that oxygen radicals produced by lipoxygenases via arachidonic acid metabolism activate NNK in human lung microsomes (22). The basic function of 5-LO is to catalyze the oxygenation of arachidonic acid and the transformation of the hydroxyperoxide intermediate to an epoxide (47). Co-oxidation of xenobiotics can occur during this metabolic process (48,49). Smith *et al.* observed a concentration-dependent increase in the rate of formation of the two NNK metabolites keto aldehyde and keto alcohol by adding soybean lipoxygenases to lung microsomal proteins (22). As shown in Table III, the addition of lipoxygenases to the microsomal incubation mixture slightly increase α-carbon hydroxylation of NNK. We incubated NNK with lipoxygenases in the absence of microsomal protein but still observed α-carbon hydroxylation of NNK. As expected, this increase in lipoxygenase-mediated NNK α-carbon hydroxylation was totally inhibited by addition of arachidonic acid or A-79175. Smith *et al.* incubated NNK with human lung microsomes and arachidonic acid without an NADPH generating system and concluded there was an arachidonic acid-supported oxidation of NNK. The only NNK metabolites included in their study were keto acid, which decreased, and keto aldehyde and alcohol, which increased compared with the control (22). We consider that our experimental conditions and results more fully support this conclusion since the microsomal 100 000 *g* fraction lacks intrinsic lipoxygenases activity (9). We incubated NNK with murine microsomal samples and arachidonic acid and did not observe microsome-mediated oxidation of NNK supported by arachidonic acid. Our metabolic analysis was extensive and included four α-carbon hydroxylation metabolites (hydroxy acid, keto acid, diol and keto alcohol), while Smith *et al.* measured only keto acid, keto aldehyde and alcohol. Detoxification of NNK by *N*-oxidation of the pyridine ring is also catalyzed by lipoxygenase. As shown in Table III, levels of lipoxygenase-mediated detoxification were lower than lipoxygenase-mediated activation. We conclude that lipoxygenases activate NNK in the presence or absence of microsomal monooxygenase and that NNK oxidation is not supported by arachidonic acid. We hypothesize that A-79175 is a more effective chemopreventive agent than MK-886 because it inhibits both cell proliferation and lipoxygenase-dependent NNK activation.

In summary, 5-LO inhibition has been shown to inhibit both lung tumor multiplicity and incidence. A combination of 5-LO and cyclooxygenase inhibitors is more effective than NSAIDs alone. The mechanism of action of inhibitors of leukotriene synthesis could be inhibition of both murine lung cell proliferation and NNK metabolic activation by lipoxygenases. Although the mechanism(s) of action remains to be clarified, 5-LO inhibitors are a promising new class of chemopreventive agents.

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