Introduction

Arachidonic acid, released from membrane phospholipids upon cell stimulation, is converted to leukotrienes by lipoxygenases or to prostanoids by cyclooxygenases. Inhibition of cyclooxygenase activity and 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-79175 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 E2 levels from 49 to 260 pg/ml and plasma LTB4 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-79175. 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.

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-79175 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 E2 levels from 49 to 260 pg/ml and plasma LTB4 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-79175. 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.

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that lipoygenases 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 lipoygenases. The role of lipoygenases 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; and (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 lipoygenases.

Materials and methods

Chemicals

NNK (99% pure by thin layer chromatography) and [5-3H]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-[4S,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), NADP+, soybean lipoygenases and sulindac were purchased from Sigma (St Louis, MO). A-79175 (99% 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 (Buchs, Switzerland) and Abbott 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 Tellyesnzyk’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 \( V = \frac{4}{3} \pi d^3 \), 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 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 reconstituted in 200 µl methanol. This extraction was repeated once. 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:0.002 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 acetone:trifluoroacetic 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 1.0 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.

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

\[ \text{Chemical structure of A-79175 and MK-886.} \]

\[ \text{Fig. 1.} \]

}\]
Inhibitors of lipoygenase

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

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Chemopreventive agent†</th>
<th>Dose of agent (mg/kg diet) (mmol/kg body wt/day)</th>
<th>Lung tumors</th>
<th>Stomach tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Multiplicity (mean ± SE) d</td>
<td>Incidence c</td>
</tr>
<tr>
<td>1 (negative)</td>
<td>None</td>
<td>None</td>
<td>0.32 ± 0.12</td>
<td>6/22*</td>
</tr>
<tr>
<td>2 (positive)</td>
<td>9.09 ± 0.02</td>
<td>None</td>
<td>8.65 ± 1.21</td>
<td>24/24</td>
</tr>
<tr>
<td>3</td>
<td>9.10 ± 0.05</td>
<td>ASA</td>
<td>294 (2.45)</td>
<td>4.83 ± 0.32*</td>
</tr>
<tr>
<td>4</td>
<td>9.09 ± 0.04</td>
<td>A-79715</td>
<td>75 (0.37)</td>
<td>2.16 ± 0.33**</td>
</tr>
<tr>
<td>5</td>
<td>9.06 ± 0.04</td>
<td>ASA + A-79715</td>
<td>294 + 75 (2.45 + 0.37)</td>
<td>1.12 ± 0.22***</td>
</tr>
<tr>
<td>6</td>
<td>9.09 ± 0.05</td>
<td>MK-886</td>
<td>25 (0.08)</td>
<td>4.16 ± 0.38**</td>
</tr>
</tbody>
</table>

*NNK was given in drinking water from week 0 to +7. Total dose was estimated to be 2.2 mmol/kg body wt.

†Feeding the chemopreventive agents was started on week –2 and pursued to the end of the assay (week +2).

†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.

§ Statistically different from negative control (group 1) (P < 0.0005).

Statistically different from negative control (group 2), χ²: P < 0.001.

† Determined from 23–96 tumors located in 14–25 mice/group, statistically different from group 2, one factor ANOVA: P < 0.005.

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 100 μl aliquot of [5,6,8,11,12,14,15-3H(N)]PGE 2 (20 000 d.p.m.) tracer was added to each sample of plasma (200 μl) mix with 5 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 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 immunosassay buffer. Two aliquots of 250 μl were used to measure 3H 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 reuspended in 150 μl ELISA buffer. 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 lipoygenases

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 μM EDTA, 3 mM MgCl₂, 10 μM [13C3]5⁻-[3H]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 sulphate and 200 μl saturated barium hydroxide. Some incubations were carried out with soybean lipoygenase (10 000 U) or with 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-79715 as a mechanism of inhibition of tumorigenesis by this agent. Incubation 8 was performed with lipoygenase co-factor under the following conditions: 8 mM EDTA, 2 mM CaCl₂, 10 μM [13C3]5⁻-[3H]NNK, 10 000 U lipoygenases 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 Millipore-LCR cartridge filter (Millipore). Five hundred microilters 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 spectrometry. 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 cm² plastic dishes and split every three days using trypsin/EDTA. Cells were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO₂.

Cell proliferation assay

Lung tumor cells were cultured on 96-well plates, at an initial concentration of 1×10⁴ cells/well with or without chemopreventive agents. Chemopreventive agents were dissolved in dimethylsulfoxide. The concentration of dimethyl-sulfoxide 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 lipoygenase metabolites were performed to determine if those metabolites are implicated in cell proliferation (Table IV). S-(SH)ETE, LTB 4, and LTB 5 in ethanol solution were added to the cells at a concentration of 7.5 μM in the presence or absence of 20 μM A-79715.

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 χ² 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.
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 mm³. ASA treatment reduced tumor volumes by 35% (P < 0.05), while MK-886 and A-79175 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 (r² = 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 2.24 ± 6% (P < 0.01), but the tumor incidence remained similar to that measured in ASA + A-79175-treated mice. More than 85% of PGE₂ and LTB₄ were extracted from plasma. Plasma LTB₄ 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 LTB₄ 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-methylisotrosmo-amino-1-(3-pyridyl)-1-butanol N-oxide result from NNK pyridine N-oxidation (20). A/J mouse lung microsomes metabolized 10 µM [5-³H]NNK by α-carbon hydroxylation (9.5%) and N-oxidation (3.9%) (incubation 1, Table III). Addition of 10,000 UI of human lipoxygenases to microsomal ³H-grown NNK (incubation 8) 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 5). 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-79175 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 totally 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

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**Table II. Plasma PGE₂ and LTB₄ levels in A/J mice after NNK treatment with or without chemopreventive agents**

<table>
<thead>
<tr>
<th>Group</th>
<th>Carcinogen</th>
<th>Chemopreventive agent</th>
<th>PGE₂ (pg/ml)b (mean ± SE)</th>
<th>LTB₄ (pg/ml)b (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>None</td>
<td>49 ± 4 (8)</td>
<td>29 ± 8 (4)</td>
</tr>
<tr>
<td>2</td>
<td>NNK</td>
<td>None</td>
<td>260 ± 68* (17)</td>
<td>71 ± 8** (5)</td>
</tr>
<tr>
<td>3</td>
<td>NNK</td>
<td>ASA</td>
<td>120 ± 35 (6)</td>
<td>n.d.</td>
</tr>
<tr>
<td>4</td>
<td>NNK</td>
<td>A-79175</td>
<td>254 ± 30* (6)</td>
<td>27 ± 4† (5)</td>
</tr>
<tr>
<td>5</td>
<td>NNK</td>
<td>A-79175 + ASA</td>
<td>96 ± 18 (6)</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>NNK</td>
<td>MK-886</td>
<td>219 ± 81 (8)</td>
<td>23 ± 4 (5)</td>
</tr>
</tbody>
</table>

*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.

**Inhibition of PGE₂ and LTB₄ synthesis**
Plasma PGE₂ 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 PGE₂. ASA reduced the NNK-induced PGE₂ plasma levels to 120 ± 35 pg/ml, a concentration similar to that measured in ASA + A-79175-treated mice. More than 85% of PGE₂ and LTB₄ were extracted from plasma. Plasma LTB₄ 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 LTB₄ back to untreated mouse levels.
the chemopreventive agents was slightly different. Significant growth inhibition of cell line 82-132 occurred with an EC₅₀ 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 EC₅₀ values were 10 µM for both MK-886 and A-79175 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. LTB₄ and LTC₄ had no effect on cell proliferation, with or without A-79175 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-79175 and MK-886 were selected according to their inhibitory ED₅₀ 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 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. This combination shows sustained improvements after a 6 month daily administration of ASA and A-79175 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 ASA use. We are the first to report the preventive efficacy of ASA against lung cancer. The preventive efficacy of ASA against lung cancer is not limited to lung tumorigenesis; Jiang et al. have inhibited benzo[a]pyrene-induced skin tumorigenesis in CD-1 mice with the ASA inhibitor TMK688 (42). Thus, inhibitors of leukotriene synthesis appear to be effective chemopreventive agents in multiple tissues.

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

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Microsomes (mg protein)</th>
<th>Lipoxygenases (inhibitor)</th>
<th>Arachidonic acid</th>
<th>Co-factor</th>
<th>α-Carbon hydroxylation (%)</th>
<th>N-oxidation (%)</th>
<th>NNK + NNAL (%)</th>
</tr>
</thead>
</table>
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

<table>
<thead>
<tr>
<th>Lipoygenase metabolite</th>
<th>Growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-79175 (mean ± SD)</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>5(S)HETE</td>
<td>116 ± 10*</td>
</tr>
<tr>
<td>LTc4</td>
<td>102 ± 7</td>
</tr>
<tr>
<td>LTB4</td>
<td>100 ± 8</td>
</tr>
</tbody>
</table>

*Statistically different from the control, Student’s t-test, P < 0.05.
Mean ± SD of three determinations for LTc4 and LTB4; five determinations for 5(S)HETE. A-79175 was added at a concentration of 20 µM. 5(S)HETE, LTc4, and LTB4 were added at a concentration of 7.5 µM.

than untreated animals (Table II). We previously reported that PGE2 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 PGE2 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 PGE2 synthesis. Induction of 5-LO by a carcinogen has never been reported. In this study plasma LTB4 concentrations more than doubled in mice bearing NNK-induced tumors. A-79175 and MK-886 returned elevated plasma LTB4 levels in NNK-treated mice to those of untreated mice and, as expected, did not affect PGE2 levels (Table II). Furthermore, MK-886 treatment did not inhibit PGE2 levels in guinea pigs (37). However, A-79175 was a more effective inhibitor of lung tumorigenesis than MK-886, despite both drugs inhibiting LTB4 synthesis to similar extents. We conclude that LTB4 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, LTB4 and LTc4 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 lipoxyn-
genases were also involved (20,22). These authors suggested that oxygen radicals produced by lipoxynagenases 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 hydroperoxide 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 lipoxynagenes to lung microsomal proteins (22). As shown in Table III, the addition of lipoxynagenes to the microsomal incubation mixture slightly increase α-carbon hydroxylation of NNK. We incubated NNK with lipoxynagenes in the absence of microsomal protein but still observed α-carbon hydroxylation of NNK. As expected, this increase in lipoxynagenes-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 lipoxynagenes 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 lipoxynagenes. As shown in Table III, levels of lipoxynagenes-mediated detoxification were lower than lipoxynagenes-mediated activation. We conclude that lipoxynagenes activate NNK in the presence or absence of microsomal monoxygenase 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 lipoxynagenes-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 lipoxynagenes. Although the mechanism(s) of action remains to be clarified, 5-LO inhibitors are a promising new class of chemopreventive agents.

Acknowledgements

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References


Inhibitors of lipoxynagenes

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