Inhibition of lung tumourigenesis by sulindac: comparison of two experimental protocols

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The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent lung carcinogen in mice and is most likely involved in the aetiology of tobacco-induced lung cancer. Two protocols using NNK and A/J mice have been developed. In the single-dose protocol, each mouse was injected once with 2 mg of NNK. In the 7-week protocol, each mouse received 9.1 mg of NNK in drinking water during 7 weeks. Mice were killed 16 weeks after NNK treatment. We observed a near-Gaussian distribution in the number of tumours per mouse in the single protocol, but not in the 7-week protocol. In the 7-week protocol, a significant number (8.6\%) of mice had more than 20 tumours/mouse. In the single-dose protocol, no mouse had more than 20 tumours. Sulindac at a dose of 123 mg/kg of diet inhibits lung tumourigenesis in the 7-week protocol, but not in the single-dose protocol. We observed that the inhibition of tumourigenesis in the 7-week protocol was proportional to the logarithm of the dose of sulindac between 15 and 123 mg/kg of diet. Treatment of mice for 7 weeks inhibits the primary humoral response to sheep red blood cells by 70\%. This observation is particularly significant considering that NNK is present in tobacco smoke and that tobacco smoking suppresses both the specific and non-specific humoral and cellular immunity. Single injections of 2.0, 3.5 or 5.0 mg of NNK had no effect on this response. Our results suggest that the immunosuppressive effects of NNK contribute to its high carcinogenic potency particularly in sustained or life-time exposure models. We hypothesize that sulindac promotes the recovery of immune system from the NNK-mediated suppression observed in the 7-week protocol. This study illustrates the importance of selecting the most appropriate protocol of carcinogen treatment in investigating the efficacies of cancer chemopreventive agents.

Introduction

Cancer chemoprevention is a multistage strategy aimed to reduce or delay the process of carcinogenesis. According to this strategy, potential agents are initially identified and tested for efficacies in in vitro model systems. The chemopreventive efficacies of agents most effective in blocking in vitro endpoints are then determined in animal tumour models (1). The selection of a responsive and sensitive animal model is crucial at that stage of the strategy. Relatively few models are available for investigating chemoprevention of lung tumourigenesis (1–3).

The nicotine-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*), is present in cigarette smoke and is a potent lung carcinogen in mice, rats and hamsters independent of the route, and type of administration (4,5). Considering its levels in tobacco smoke and its carcinogenic potency, Wynder and Hoffmann suggested that NNK plays a causative role in the development of smoking-related lung adenocarcinomas (6,7). The use of NNK in the induction of lung tumours in laboratory animals is particularly relevant to human lung cancer. The metabolism, DNA and protein alklylation by NNK are well documented (5,8). However its effects on the immune system have not been investigated.

Two experimental protocols have been developed in A/J mice to assess the efficacies of natural and synthetic chemopreventive agents. A single intraperitoneal injection of NNK at a dose of 2 mg per A/J mouse is sufficient to induce an average of 10–12 lung adenomas per mouse (9). A significant number of these adenomas progress with time to adenocarcinomas (10). Hecht et al. suggested that this protocol for lung tumour induction with NNK could be useful for determining the efficacies of chemopreventive agents and their involvement in preventing the initiation or promotion stages of carcinogenesis (9). Various investigators have used this single dose protocol to demonstrate the efficacies of 4-ipomeanol, diallyl sulphide, indole-3-carbinol, citrus fruit oils, arylalkyl isothiocyanates and tea extracts against lung tumourigenesis (11–16). A second model using the same carcinogen and strain of mice was developed in our laboratory. According to this protocol, mice are given NNK in the drinking water during 7 weeks (17). The numbers of lung adenomas per mouse average 8.4 (18). Using this protocol, we demonstrated the efficacies of ellagic acid, butylated hydroxyanisole and various non-steroidal anti-inflammatory drugs (NSAIDs) in the inhibition of lung tumour development (18–20).

The aims of this study were to compare the statistical distribution of lung adenoma multiplicities and to compare the chemopreventive efficacies of sulindac in the above-described protocols. The advantages and limitations of the two protocols in chemoprevention studies are discussed and the effects of the two protocols on the primary humoral response are compared.

Materials and methods

Chemicals

NNK was purchased from Chemsyn Science Laboratories, Lenexa, KS. Its purity was higher than 98\%. Sulindac (99.16\% purity) was a generous gift from Merck Frosst Canada Inc. Guinea-pig complement was obtained from Cedarlane Laboratories (Hornby, Canada).

Animals

Female A/J mice (6–7 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). They were housed in groups of five per cage and kept under standard conditions (21 ± 1°C, 40 ± 5\% relative humidity; 12 h:12 h light-dark cycle). The mice were sorted out on weight basis in groups of 10, 20 or 25 mice, and given tap water and powdered diet ad libitum.

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**SINGLE DOSE PROTOCOL**

**Fig. 1.** Experimental design of the single-dose protocol [according to Hecht et al. (9)].

**SEVEN WEEK PROTOCOL**

**Fig. 2.** Experimental design of the 7-week exposure protocol.

### Diets

Mice were fed with a powdered AIN-76A semi-purified diet purchased from Teklad Premier Laboratory Diets (Madison, WI). It contained 50% sucrose, 20% purified casein, 15% corn starch, 5% fibre, 5% corn oil, 3.5% mineral mix, 1% vitamin mix, 0.3% tβ-methionine and 0.2% choline bitartrate. Sulindac was mixed with the diet for 8 min in a V-shaped blender. Dietary standard error was 0.29.

### Animal treatments

In the single-dose protocol, illustrated in Figure 1, NNK was dissolved in 0.9% sterile NaCl at a concentration of 2 mg/0.5 ml. Three groups of 20, 25 and 25 mice were injected i.p. with 2 mg (10 µmol) of NNK per mouse. They were fed with AIN-76A diet starting two weeks before injection of NNK and until sacrifice at +16 weeks (Groups 1–3). Two groups were given sulindac in the diet (123 mg/kg) either before NNK treatment (group no. 2) or throughout (group no. 3) the bioassay (Table I).

In the 7-week exposure protocol, illustrated in Figure 2, a stock solution of NNK was prepared in distilled water at a concentration of 3.74 mg/ml. NNK was administered in the drinking water at an initial concentration of 62.4 µg/ml and was adjusted thereafter for each cage according to water consumption. The cumulative dose of NNK was 9.1 mg per mouse. Four groups of 25 mice received a mixture of AIN-76A diet and sulindac at concentrations of 15, 30, 61 or 123 mg/kg of diet (Figure 4). The diets were given starting two weeks before NNK treatment and throughout the experiment (25 weeks). One control group of 10 mice was not treated with NNK and was given AIN-76A diet (group no. 4, Table I). Two groups of 25 mice were given NNK in drinking water and received AIN-76A diet with (group no. 6) or without (group no. 5) sulindac at a concentration of 123 mg/kg of diet. Mice were killed 23 weeks after starting NNK treatment in drinking water.

Lungs and major organs were fixed in Tellyesniczky’s fixative for 7 days before counting the number of surface adenomas under a dissecting microscope as described previously (21). The stomachs were fixed in situ with 0.5 ml of 10% formalin, excised and stored in formalin. Papillomas larger than 1 mm were counted.

### Stability of sulindac in the diet

Diet samples containing 123 mg of sulindac/kg were collected before feeding or at the end of a 4-day period of feeding. Two g samples of diet were extracted with 20 ml of acetonitrile: 0.01 N HCl (9:1). A 100 µl aliquot of indomethacin (100 µg/ml acetonitrile) was added as internal standard to 2 ml of supernatants. Samples were evaporated to dryness and suspended in 200 µl of HPLC mobile phase. The extraction was repeated once. Sulindac stability was determined by reverse phase HPLC on a C-18 Bondapak column (Waters Associates, Milford, MA). The mobile phase consists of acetonitrile: KH₂PO₄ 50 mM, pH 3.7 (55:45, v/v). Elution at a flow rate of 1 ml/min was monitored at 313 nm. Results were calculated from linear regression curve relating peak area to sulindac concentrations.

### Direct haemolytic plaque-forming technique

Groups of five A/J female mice, 7 weeks old, were injected i.p. with a single dose of 2.0, 3.5 or 5.0 mg of NNK dissolved in saline. Control mice were injected 200 µl of normal saline. Three days later, mice were injected i.p. with 5×10⁸ fresh sheep red blood cells (SRBCs) in 200 µl of saline solution. Four days later, spleens were removed and teased through a 70-µm nylon mesh into Hank’s balanced salt solution. A 100-µl aliquot of a 1:40 dilution of the splenocyte suspensions and 100 µl of SRBCs (3×10⁷/ml) were added to 1 ml solution of 0.7% agarose. Cells were plated and incubated at 37°C for 90 min. One ml of a 1:10 dilution of guinea-pig complement was added and the incubation continued for another 90 min. The number of cells secreting anti-SRBC IgM antibodies was determined in triplicate (22).

### Statistical analysis

Normality was determined with the Shapiro–Wilk test. The distribution in the single dose protocol had a p value of 0.0004, skewness value of 0.6 and kurtosis factor of 0.4. In the 7-week exposure model the distribution had a P value of 0.0001, a skewness value of 2.6 and kurtosis factor of 8.9. In the 7-week exposure model, the skewness value was 0.1 and the kurtosis factor was 0.6 after logarithm transformation.

### Results

The distribution of lung tumour multiplicities in mice receiving a single-dose of NNK is shown in Figure 3. A total of 137 mice from three experiments were included in this analysis. The distribution follows a near-normal Gaussian distribution.

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Inhibition of tumourigenesis by sulindac

Table I. Efficacy of sulindac in inhibiting NNK-induced lung tumourigenesis in A/J mice

<table>
<thead>
<tr>
<th>Group no.</th>
<th>No. of mice at risk</th>
<th>NNK dose (mg/mouse)</th>
<th>Protocol</th>
<th>Time of feeding sulindac</th>
<th>Long tumour multiplicity</th>
<th>Tumour incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>2</td>
<td>Single dose</td>
<td>None</td>
<td>9.88 ± 1.06</td>
<td>25/25</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>2</td>
<td>Single dose</td>
<td>–2 → 0</td>
<td>9.60 ± 1.02</td>
<td>20/20</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>2</td>
<td>Single dose</td>
<td>–2 → +16</td>
<td>8.37 ± 0.88</td>
<td>24/24</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>0.10 ± 0.10</td>
<td>1/10</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>9.1</td>
<td>7-week</td>
<td>None</td>
<td>10.88 ± 1.88</td>
<td>25/25</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>9.1</td>
<td>7-week</td>
<td>–2 → +23</td>
<td>5.96 ± 0.99*</td>
<td>24/24</td>
</tr>
</tbody>
</table>

Sulindac was given at a dose of 123 mg/kg diet (12.0 mg/kg body weight). Mean ± SE Statistically different from value observed with group 5. Student’s t-test. *P < 0.05

Fig. 3. Distribution of the number of mice according to the number of tumours/mouse in the single-dose and 7-week exposure protocols.

The extend of inhibition of gastric tumourigenesis is also related to the dose of sulindac. The incidences of forestomach tumours were 20, 16, 8, 4 and 4% at doses of 0, 15, 30, 61 and 123 mg/kg of diet of sulindac, respectively (data not shown). Feeding mice with 123 mg/kg sulindac reduced the number of lung tumours to 5.96 tumours/mouse (45% inhibition). The multiplicity of lung tumours was inversely proportional to the logarithm of the concentration of sulindac in the diet.

Fig. 4. Inhibition of NNK lung tumorigenesis by sulindac at four different concentrations. NNK was administered to A/J mice according to the seven-week protocol. Values are mean ± SE from 25 mice. Curve was fitted by least-square method, and data were analysed by linear regression ($r^2 = 0.999$).

Table II. PFC responses to SRBCs after treatment with NNK

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Dose of NNK mg/mouse</th>
<th>Route</th>
<th>PFC/million cells (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-dose</td>
<td>None (control)</td>
<td>i.p.</td>
<td>293 ± 42</td>
</tr>
<tr>
<td>Single-dose</td>
<td>2</td>
<td>i.p.</td>
<td>313 ± 33</td>
</tr>
<tr>
<td>Single-dose</td>
<td>3.5</td>
<td>i.p.</td>
<td>316 ± 20</td>
</tr>
<tr>
<td>Single-dose</td>
<td>5</td>
<td>i.p.</td>
<td>318 ± 24</td>
</tr>
<tr>
<td>7-week</td>
<td>9.1</td>
<td>p.o.</td>
<td>92 ± 32 *</td>
</tr>
</tbody>
</table>

*This group received 200 µl of normal saline. Statistically different from the four other groups by Dunnett’s-test ($P < 0.01; n = 5$).

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Primary humoral response to SRBCs was determined in A/J mice treated with NNK (Table II). Single injections of 2.0, 3.5 or 5.0 mg of NNK did not reduce the plaque-forming cells (PFC) response. A dose of 10 mg causes death and significant weight lost. In contrast, a 70%-inhibition of the PFC response was observed after a seven-week treatment with a cumulative dose of 9.1 mg NNK.

Discussion

The A/J mouse has been used extensively to determine the carcinogenic potency of synthetic and natural compounds.
(reviewed in ref. 23). This strain is particularly susceptible to develop adenomas that progress with time to carcinomas within adenomas and then to carcinomas (24). The morphological characteristics of mouse lung carcinomas are reminiscent of adenocarcinomas observed with increased frequency in smokers (25). Various protocols have been developed to treat A/J mice with carcinogens (3,9,17,21,23,26). The most common route of administration is the i.p. route. The carcinogenic response of some carcinogens is higher with the i.p. route than by gastric intubation (27). Our results show that NNK is more carcinogenic by the i.p. route than by oral administration. This is in agreement with the study of Hecht et al. reporting a higher lung tumour multiplicity following i.p. administration of NNK (3). The single-dose protocol documented in this paper was developed by Hecht et al. (9). This protocol has the advantages of being safer since carcinogens are handled only once. In this study, we have demonstrated that tumour multiplicity when using this single-dose protocol has a near-normal Gaussian distribution which simplifies statistical analysis of the data (Figure 3). Hecht et al. suggested that the single-dose protocol could be applied to study selectively the initiation and promotion phases of chemicals. However, no promoter have ever been investigated. We observed that feeding A/J mice with butylated hydroxytoluene after a single NNK injection increased the lung tumour response two-fold (unpublished data). The single-dose model has proven to be useful to assess the chemopreventive efficacies of agents able to block P450-mediated activation of NNK. For instance, isothiocyanates administered shortly before i.p. injection of NNK inhibit P450 activity, NNK bioactivation, NNK-DNA adduct formation and prevent tumour development in A/J mice (28,29). In this study, we did not observe any inhibition of lung tumourigenesis in mice fed sulindac prior to NNK injection. This observation indicates that sulindac and its metabolites are not effective inhibitors of the activation of NNK given in bolus doses. This contrasts with our previous observation that the sulphide metabolite of sulindac inhibited the bioactivation of NNK in mouse lung explants cultured in vitro (30).

The rational for developing a second protocol with NNK was to expose mice to this tobacco-specific carcinogen at relatively low dose for an extended period of time, thus simulating the exposure of smokers to NNK. The levels of NNK in the smoke of one cigarette range from 10 to 200 ng per cigarette and tobacco smoking could last decades (31). As expected, a larger cumulative dose is required in this 7-week protocol to induce a tumour response comparable to that observed in the single-dose model. However, the daily dose of NNK is a relatively low and is estimated to be 186 µg. In this study, we observed that the multiplicities showed a non-symmetrical distribution from the mean number of tumours/mouse. Although we do not know the reasons for such a distribution, it could reflect a larger consumption of NNK by mice having dominant behavioural characteristics. In our protocol, five mice were housed per cage. Administration (p.o. or i.p.) of small repetitive subdoses of NNK could be an alternative to this protocol (3). As expected from the DNA-alkylation data, the tumourigenic responses in the single-dose and 7-week protocols are not linear with the dose of NNK (8,19).

We were the first to demonstrate that sulindac, at a dose of 123 mg/kg of diet, prevents lung tumourigenesis. A 53% inhibition of lung tumourigenicity was observed using the 7-week protocol (32). The results of this initial study were confirmed by two subsequent studies showing a 51 and 52% inhibition at the same dose of sulindac (18,33). In this study, we observed a 45% inhibition of lung tumourigenesis with the same concentration of sulindac. The inhibition of tumourigenesis was related to the logarithm of the dose of sulindac (Figure 4). This observation further supports the hypothesis that sulindac could be effective in preventing lung carcinogenesis induced by NNK in tobacco smokers. The daily dose of sulindac recommended is 400 mg corresponding to 5.7 mg/kg body weight and to one-half of the dose (12 mg/kg body weight; 123 mg/kg diet) used in this study. No epidemiological studies have investigated specifically the protective effect of sulindac intake and lung cancer risk in smokers. However, Dehemachers and Everson observed a reduced incidence of lung cancer associated with increased aspirin use (34). Aspirin and sulindac are both anti-inflammatory agents. The protective effect of sulindac against colon carcinogenesis in human and animals is well documented (35,36).

In this study, we reported for the first time that NNK is immunosuppressive. This observation is particularly significant considering that NNK is present in tobacco smoke and that tobacco smoking is suppressing the specific and non-specific humoral and cellular immunity (31,37,38). The immunosuppression observed with NNK is to some extent similar to that observed with other nitrosamines. Wayneforth and Magee reported that a single dose of N-nitrosodimethylamine does not reduce the number of spleen PFC in mice or in rats (39). A single dose of N-nitrosodimethylamine has no effect on the B lymphocyte response, but suppresses the T-B cell collaboration (40). Desjardins et al. concluded that N-nitrosodimethylamine suppresses the IgM antibody response to SRBC in mice in a time- and dose-related manner (41). The immunotoxic potency of dialkylnitrosamines is inversely related to aliphatic chain length (42). NNK is a non-symmetrical nitrosamine with a methyl group as in N-nitrosodimethylamine and a four-carbon alkyl chain such as in the dialkylnitrosamines. Our results suggest that the immunosuppressive effects of NNK are contributing to its high carcinogenic potency particularly in sustained or life-time exposure models developed by various investigators (43–46). We hypothesized that sulindac is helping the immune system to recover from the NNK-mediated suppression observed in the 7-week protocol, this attenuation in the immunotoxic response being mediated by PGE_2. In a recent study, we observed that a 7-week treatment with NNK increased plasma levels of PGE_2 and this increase was attenuated by sulindac in a dose-related manner (47). The inhibition of PGE_2 synthesis by sulindac and other NSAIDs and the immunosuppressive properties of PGE_2 are well documented (48–50). All data obtained in our laboratory are consistent and support the hypothesis that lung cancer chemopreventive activities of NSAIDs is related to their capacities to inhibit PGE_2 synthesis. This hypothesis provides some rationale for the absence of inhibition of tumourigenesis by sulindac in the single-dose protocol since acute exposure to NNK is not immunosuppressive. Consistent with this hypothesis, indomethacin had no effect on the growth of lung tumours induced by a single dose of NNK (26). It should be mentioned that the significance of the inhibition of PGE_2 synthesis as a mechanism of chemoprevention of colon cancer by sulindac is controversial (51–54).
Inhibition of tumourigenesis by sulindac


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