Inhaled Isotretinoin (13-cis Retinoic Acid) Is an Effective Lung Cancer Chemopreventive Agent in A/J Mice at Low doses: A Pilot Study

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ABSTRACT

In previously treated head-and-neck cancer patients, p.o. administered isotretinoin (13-cis retinoic acid) reduced the occurrence of second aerodigestive tumors, including lung tumors, but side effects made chronic therapy problematic. We reasoned that inhaled isotretinoin might provide sufficient drug to the target cells for efficacy while avoiding systemic toxicity, and we proceeded with the pilot study reported here. Male A/J mice were given single i.p. doses of urethane, a common experimental lung carcinogen, or benz[a]pyrene (BaP) or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK), putative major carcinogens in tobacco smoke. The following day, exposures to isotretinoin aerosols for 45 min daily at 1.3, 20.7, or 481 μg/l were initiated. After 2 weeks, the high dose caused severe toxicity on the snout skin, necessitating a reduction of dose frequency to twice a week. As a precaution, the mid dose was reduced to three exposures per week. The weekly total deposed doses after the dose frequency reductions were calculated to be 0.24, 1.6, and 24.9 mg/kg for the low, mid, and high doses, of which 16% was estimated to be deposited in the lungs. The weekly deposited pulmonary drug doses were calculated to be 0.01, 0.07, and 1.1% of a previously reported ineffective oral dose in urethane-treated A/J mice. After 10–16 weeks, mice were sacrificed to count areas of pulmonary hyperplasia and adenomas. For all carcinogens, the mice exposed to the high isotretinoin dose showed reductions of tumor multiplicity ranging from 56 to 80% (P < 0.005) in BaP- and NNK-treated mice, respectively, and was tolerated until ~12 weeks, when both these and the high-dose mice began losing weight. The low-dose mice had nonsignificant reductions of 30% (P < 0.13) and 16% (P < 0.30) for BaP- and NNK-treated mice, respectively without any evidence of side effects. For BaP- and NNK-treated mice, numbers of hyperplastic areas directly correlated to dose level and inversely to tumor number, suggesting arrested progression. Inhaled mid-dose isotretinoin caused up-regulation of lung tissue nuclear retinoic acid receptors (RARs) relative to vehicle-exposed mice, RARα (3.9-fold vehicle), RARβ (3.3-fold), and RARγ (3.7-fold), suggesting that these receptors may be useful biomarkers of retinoid activity in this system. The encouraging results from this pilot study suggest that inhaled isotretinoin merits evaluation in people at high risk for lung cancer.

INTRODUCTION

Former and current smokers, as well as individuals who have been successfully treated for a first aerodigestive cancer, would greatly benefit from drugs that prevent progression of lung neoplasia. This vast group of people comprises more than one-third of the adult population of the United States, all sharing significant risk for developing lung cancer. Retinoids are necessary for the maintenance of respiratory epithelial cell differentiation in vivo and can induce terminal differentiation or apoptosis of initiated epithelial cells, and thus have prospects as preventive agents for some forms of cancer. Furthermore, vitamin A deficiency has been shown to increase the number of BaP2-DNA adducts in cultured hamster trachea (1).

In a randomized clinical trial, the oral administration of isotretinoin (1–2 mg/kg/day) was significantly protective against second aerodigestive tumors in a cohort of previously treated head-and-neck cancer patients (2, 3). Because of the effectiveness of isotretinoin as a preventative of some forms of cancer, its efficacy as a lung cancer chemopreventive agent is under study in several clinical trials (Protocol IDS: UCHSC-92382, NCI-V94-0506 and CBRG-9208, NCI-V92-0159, NBSG-9208).

Enthusiasm for the use of isotretinoin as a chemopreventive agent has been held back, in part due to the occurrence of debilitating drug side effects associated with the doses used in the M. D. Anderson study (4), which, based on pharmacokinetic

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2 The abbreviations used are: BaP, benz[a]pyrene; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane; MMAD, mass median aerodynamic diameter; RAR, retinoic acid receptor; TBS, Tris-buffered saline; COX, cyclooxygenase.
data, provided steady-state blood levels of 100–200 ng/ml. Sixteen of the 49 patients in this head-and-neck chemoprevention trial did not complete the course of therapy. Because the benefits of isotretinoin treatment is reduced after cessation of treatment (3), the expectation is that chronic drug administration would be required, making the patient compliance issue critical.

To address the toxicity concerns, investigators have contemplated lowering the dosages of the drug, but it is unclear whether such a change would jeopardize the desired therapeutic effect. Oral doses of 1 mg/kg failed to reverse lung metaplasia in smokers (5). Attempts to lessen the severity of the toxic effects by coadministration of vitamin E are under study in clinical trials (Protoc IDS: MDA-DM-97078, NCI-P98-0132), and clearly additional work is merited in this critical area.

We reasoned that because lung cancer arises in the lung epithelium, direct application to the target cells would improve the therapeutic index. Aerosol inhalation can deposit drug directly on the population of cells caught up in the early phase of cancer, potentially achieving much more efficiency compared with reliance on diffusion from the blood. There are theoretical bases to expect major differences in potency between oral and inhaled retinoids. Some highly lipophilic compounds can be significantly retarded in their clearance from the lung epithelial surface into the blood stream (6, 7). Because the reverse also is probably the case, the poor results with oral administration may simply be a case of too little drug reaching the target cells in some parts of the lung. In addition, isotretinoin is avidly bound by serum albumin, limiting its availability for promotion of differentiation and inhibition of proliferation (8). Direct application to the lung epithelium may avoid much of the protein binding, thus greatly increasing potency at the target site.

Surprisingly, despite longstanding interest in isotretinoin, information on its in vivo pharmacology as a cancer preventive agent in animal models is scarce. In one study, p.o. administered isotretinoin of $>300$ mg/kg weekly failed to prevent urethane-induced lung cancer in the A/J mouse model (Ref. 9; Table 1). Despite this failure, we felt that direct application to the lung epithelium merited evaluation. To test this premise, we elected to expose carcinogen-treated A/J mice to isotretinoin aerosol for this pilot study.

### MATERIALS AND METHODS

#### Lung Carcinogenesis Model

The A/J mouse is a well-established animal model for preclinical chemoprevention studies (10, 11). This strain has a hereditary predisposition for lung cancer, the so-called pulmonary adenoma susceptibility (Pas) genes. A strong candidate for one of these genes, Pas-1, is the K-ras proto-oncogene (12). Carcinogenesis in this model with NNK as the inciting agent has been studied; therefore, the times required to develop hyperplastic areas, adenomas, and carcinomas are well known (13). In addition, the timing of molecular changes associated with carcinogenesis has been studied (14) and is similar to those in humans (15). In both species, K-ras mutations are common early events.

#### Experimental Design

Mice received injections of one of three carcinogens and were exposed by inhalation in groups of 21 to three graded concentrations of isotretinoin or vehicle for 10 (urethane-treated mice) or 16 (NNK- and BaP-treated mice) weeks. Forty-six mice treated with each carcinogen and 46 untreated controls were maintained in cages and were not exposed. Some of these were sacrificed at intermediate times to determine the progress of carcinogenesis. At first, exposure was daily for all doses, but after 12 days it was reduced to twice weekly for the highest dose because of severe local toxicity and three times weekly for the middle dose as a precautionary measure (Table 2).

#### Animals and Treatment

Male A/J mice (Jackson Laboratories), 6–8 weeks old, were quarantined a minimum of 7 days. Their diet throughout the experiment was AIN-76A, which, for NNK, gives higher tumor counts than NIH-07 (16). At 9–10 weeks of age, the mice received single 0.2-ml doses (20 mg of urethane in saline, 0.6 mg of NNK in saline, or 2 mg of BaP in tricaprylin) by i.p. injection. Daily 45-min exposures to isotretinoin aerosol or vehicle were started the next day.

#### Formulation of Nebulizer Solution

Powdered isotretinoin was dissolved in 100% ethanol plus 0.1% α-tocopherol and 0.1% ascorbyl palmitate to give isotretinoin concentrations ranging from 0.1 to 10 mg/ml. The formulations were prepared monthly. The solutions were protected from light and stored at $-5^\circ$C until use. UV-visible spectrophotometric verification of the formulated test article concentration was performed on all batches in advance of inhalation treatment with the test article solution. Only formulations within ±10% of the targeted concentration were used on study.

#### Inhalation Exposure

Solutions were aerosolized using a Pari LC-plus nebulizer (Pari, Richmond, VA). Animals were exposed in nose-only exposure units designed to provide a fresh supply of the test atmosphere to each animal, independent from other animals. The exposure units were based on the design described by Cannon et al. (17). The units consisted of multi-tier modular sections, each tier containing eight exposure ports located peripherally around a central delivery plenum.
et al. monodisperse aerosol (24). Note: Reported minute volumes for mice range from slightly less than the Guyton value used here to slightly greater than the mean tumor incidence of the treatment and the control groups was determined using the Mann-Whitney Rank Sum test. The lungs were evaluated in a blinded fashion so that neither carcinogen nor isotretinoin dose levels were known to the evaluator, who visually counted hyperplastic areas and adenomas on the lung pleural surface as described previously (18–20). The significance of the differences between the mean tumor incidence of the treatment and the control groups was determined using the Mann-Whitney Rank Sum test (Statmost TM; DataMost Corp., Sandy, Utah).

### Apparatus and Reagents for Western Blot Analysis
The X cell II Mini-cell and Blot Module was used with 10% Tris-glycine gels and transfer buffer and Tris-glycine SDS sample buffer; Tris-glycine SDS was used as running buffer (NOVEX-NOVEL Experimental Technology Inc., San Francisco, CA).

### Experimental Design
Five lungs each from the urethane-treated vehicle, low-dose, and mid-dose animals were snap frozen in liquid nitrogen and stored at −70°C for determination of RARα, β, and γ by Western blot analysis. In addition, five lungs each from urethane-injected unexposed mice and untreated control mice were designated for Western blots. The lungs were homogenized, and the RARs were determined by standard Western blotting (21, 22). Because of deaths early in the high-dose experiment, no lungs were available for Western blot analysis for the high-dose exposures, i.e., all tissue was used for lesion quantitation purposes.

### Samples
Lung tissue was collected, frozen on dry ice, and kept at −70°C until used. A 500-mg portion was diced in small pieces and homogenized in 300 μl of cold PBS with a hand-held homogenizer for 2–3 min. Samples were centrifuged at 500 rpm for 5 min or until the supernatant was clear. The pellet was suspended in 400 μl of cold buffer A [2 μl of 0.5 M EDTA, 10 μl of 1 mM EGTA, 50 μl of 100 mM phenylmethylsulfon fluoride, 10 μl of 1 mM DTT, and 10 ml of 10 mM HEPES (pH 7.9) + 10 mM KCl], and left at ice temperature for 15 min. A 25-μl volume of a 10% solution of NP-40 was added, and the samples were mixed in a vortex vigorously for 10 s. Samples were centrifuged at 14,000 rpm for 1 min at 4°C, and the pellet was treated once more in this fashion. The supernatant was removed, and 25–100 μl of buffer C [4 μl of 0.5 M EDTA, 20 μl of 100 mM EGTA, 20 μl of 0.1 M phenylmethylsulfon fluoride, 2 μl of 1 mM DTT, and 2 ml of 20 mM HEPES (pH 7.9) + 0.4 M NaCl] were added. Pellets were resuspended by tapping gently on the bottom of the Eppendorf tube. Samples were rocked vigorously in a bucket of ice on an orbital shaker for 30 min. Samples were then centrifuged at 14,000 rpm for 5 min at 4°C. Supernatants were kept frozen at −70°C until needed. Protein concentrations were determined using the Bradford method (23).

### Analysis
Samples were prepared by adding one part sample buffer to one part sample and mixing well. For denatur-

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### Table 2  Isotretinoin doses in pilot efficacy studies

<table>
<thead>
<tr>
<th>Exposure level</th>
<th>Mean aerosol concentration (μg/l)</th>
<th>Pulmonary deposited Isotretinoin dose/ Exposure daya (μg/kg)</th>
<th>Total deposited Isotretinoin dose/ Exposure dayb (μg/kg)</th>
<th>Weekly pulmonary dose (mg/kg)</th>
<th>Weekly total dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>1.3</td>
<td>5.2</td>
<td>33.6</td>
<td>0.037</td>
<td>0.235</td>
</tr>
<tr>
<td>Mid</td>
<td>20.7</td>
<td>83</td>
<td>535</td>
<td>0.582</td>
<td>0.75</td>
</tr>
<tr>
<td>High</td>
<td>481</td>
<td>1931</td>
<td>2400</td>
<td>13.5</td>
<td>87.0</td>
</tr>
</tbody>
</table>

### Notes:
- a Aerosol concentration, (μg/l) × (2.1 × 22g<sup>−7/2</sup>) ml/min × 1 l/1000 ml × 45 min × 0.022 kg × 0.092, where (2.1 × 22g<sup>−7/2</sup>) is the Guyton formula for minute volumes, in ml/min, of a 22-g mouse (25), and 0.092 is the pulmonary deposition fraction of a 1.09 μm (aerodynamic diameter) monodisperse aerosol (24). Note: Reported minute volumes for mice range from slightly less than the Guyton value used here to slightly greater than 2-fold (49), but because Ruabe et al. (24) based fraction of deposition on the Guyton formula, it is appropriate to use it here as well.
- b Calculated by substituting total deposition fraction (0.592; Ref. 24) in place of the pulmonary deposition (0.092) in footnote a.
- c Low, exposed daily throughout; mid and high, exposed daily during the first 12 days and 3-weekly thereafter.

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During exposures, animals were restrained in unstoppered polycarbonate tubes (C&H Technologies, Westwood, NJ) through which a flow of aerosol, 350–500 ml/min per mouse, passed from the chamber. Each tube was tapered at one end to approximately fit the shape of the animal’s head, and the diameter of the cylindrical portion of the cone was such that the animal could not turn in the cone. Each cone was fastened to the inhalation chamber with the nose portion of the cone protruding through a gasket into the chamber, permitting the animal to breathe the test or control atmosphere emanating from within the central plenum.

### Aerosol Characterization
To determine aerosol concentrations, measured volumes of aerosol were drawn through filters, which subsequently were analyzed for isotretinoin by a UV-visible method. To determine particle size, aerosol was drawn through Mercer-type cascade impactors (InTox, Albuquerque, NM) equipped with filters on each stage and a backup filter. The individual filters were analyzed for isotretinoin and the MMADs and geometric standard deviations were calculated from the data using Battelle software.

### Quantitation of Lung Lesions
Within 24 h of the last inhalation exposure, animals were euthanized by i.p. injection of pentobarbital, and their lungs were removed and fixed in Bouin’s solution or flash frozen for RAR determination. The lungs were evaluated in a blinded fashion so that neither carcinogen nor isotretinoin dose levels were known to the evaluator, who visually counted hyperplastic areas and adenomas on the lung pleural surface as described previously (18–20). The significance of the differences between the mean tumor incidence of the treatment and the control groups was determined using the Mann-Whitney Rank Sum test (Statmost TM; DataMost Corp., Sandy, Utah).

### Biomarkers: RAR Induction
#### Antibodies
Polyclonal antibodies to RARα, β, and γ (Santa Cruz Biotechnology Inc., San Francisco, CA) were used with a BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit; Boehringer Mannheim Corporation, Indianapolis, IN).
Inhaled Isotretinoin

RESULTS

molar concentrations and SDs were 1.3
0.6
1.2
m
0.005, 0.081, and 2 mg/kg per exposure. Ca-
1.09 μm
9.2 ± 0.9
2.1 × [mass (g)]^{0.75} ml/min (25). Assuming the same deposition effi-
31.4 ± 1.6
29.2 ± 2.2
29.3 ± 1.5
32.2 ± 2.4
15.9 ± 1.9^b
6.5 ± 0.5
3.8 ± 0.5
4.1 ± 0.6
3.6 ± 0.3
4.5 ± 0.7
4.5 ± 0.7
14.7 ± 1.1
9.2 ± 0.9
7.2 ± 0.8
6.1 ± 0.5^d
5.2 ± 1.0^d

\[ \frac{1}{3} \], where \( d_{\text{final}} \) is the final diameter, \( d_{\text{orig}} \) is the original diameter, and \( f \) is the mass fraction of solute.]

Calculations of Deposited Dose. Inhaled monodisperse particles having an aerodynamic diameter of 1.09 μm deposit 9.2% in the pulmonary region, with 59.2% total deposition (24). Taking the average mouse weight as 22 g, the respiratory minute volume, calculated as Raabe \( \text{Raabe} \) (24) had done, was 2.1 × [mass (g)]^{0.75} ml/min (25). Assuming the same deposition efficiency as 1.09-μm monodisperse particles (for simplicity; the actual values would vary somewhat for these aerosols), the calculated daily pulmonary doses of isotretinoin for each 45-min exposure were ~0.005, 0.081, and 2 mg/kg per exposure. Calculated total deposited doses were 0.034, 0.54, and 12.4 mg/kg per exposure (Table 2).

Chemosuppression by Inhaled Isotretinoin. All comparisons are to the vehicle-exposed control mice unless noted. Mice exposed to the high isotretinoin dose had substantial reductions in tumor multiplicity, ranging from 56 to 80% below vehicle controls, for all three carcinogens (Table 3), but during daily exposures for the first 2 weeks, they experienced excessive toxicity to the snout and forelimbs. These mice lost weight (Fig. 1), and ~35% died. Following a 2-day respite, the exposure frequency was reduced to twice weekly, and the body weights increased to those of the vehicle-exposed control mice (Fig. 1) and the lesions resolved, although two more mice died early in the study (Table 4). At the end of exposure, weights were again below those of the vehicle controls (Fig. 1). In light of the significant consequences of local retinoid toxicity in this model, extrapolation of these results to humans will be difficult.

Table 3  Lung tumorigenesis inhibition in A/J mice

<table>
<thead>
<tr>
<th>Carcinogen</th>
<th>Weekly 13-cis pulmonary dose,^a (mg/kg)</th>
<th>Tumors per lung set (mean ± SE)</th>
<th>Hyperplastic areas per lung set (mean ± SE)</th>
<th>Total lesions per lung set (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urethane</td>
<td>Cage control</td>
<td>29.9 ± 1.5</td>
<td>1.5 ± 0.3</td>
<td>31.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Vehicle control</td>
<td>24.2 ± 1.9</td>
<td>4.6 ± 0.9</td>
<td>29.2 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>25.7 ± 1.7</td>
<td>3.6 ± 1.1</td>
<td>29.3 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>26.4 ± 2.1</td>
<td>5.9 ± 1.2</td>
<td>32.2 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>10.6 ± 1.2^a</td>
<td>5.3 ± 1.3</td>
<td>15.9 ± 1.9^b</td>
</tr>
<tr>
<td></td>
<td>NNK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cage control</td>
<td>1.9 ± 0.3</td>
<td>4.5 ± 0.5</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Vehicle control</td>
<td>2.5 ± 0.4</td>
<td>1.3 ± 0.3</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>2.1 ± 0.3^a</td>
<td>2.0 ± 0.4</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.3 ± 0.2^b</td>
<td>3.3 ± 0.3^b</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>0.8 ± 0.3^b</td>
<td>3.8 ± 0.6^b</td>
<td>4.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>BaP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cage control</td>
<td>12.8 ± 1.1</td>
<td>1.9 ± 0.4</td>
<td>14.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Vehicle control</td>
<td>9.0 ± 1.0</td>
<td>0.2 ± 0.1</td>
<td>9.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>6.4 ± 0.7^f</td>
<td>0.8 ± 0.3</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>3.0 ± 0.5^g</td>
<td>3.2 ± 0.4^b</td>
<td>6.1 ± 0.5^d</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>1.8 ± 0.5^g</td>
<td>3.3 ± 0.9^b</td>
<td>5.2 ± 1.0^d</td>
</tr>
</tbody>
</table>

^a Rounded from Table 2 values for weeks 3–16.
^b–d Compared with vehicle control: ^b P < 0.005; ^c P < 0.005; ^d P < 0.05.
^ef Trend for fewer tumors; ^e P < 0.30 compared with vehicle control; ^f P < 0.13 compared with vehicle control.

ing conditions, samples were heated at 95°C for 5 min. Five μl of Rainbow Standard and 5 μl of Biotinylated Molecular Marker were used. Twenty μg of protein per sample was loaded in each lane. Electrophoresis was performed with the voltage set at 125 V for 1–1.5 h. Gel transfer was executed at 25 V for 2 h. The membrane was stained in Ponceau S for 5 min and was incubated in 1 ml of blocking solution and 9 ml of TBS for 60 min and with primary antibody solution overnight (20 μl of primary antibody solution in 1 ml of blocking solution and 9 ml of TBS; dilution of 1:500). The membrane was washed in PBS-Tween 20 three times for 10 min each, and then was incubated in 1 ml of blocking solution and 19 ml of TBS along with 20 μl of antibiot horseradish peroxidase-linked antibody and 2 μl of secondary (rabbit antimouse antibody; 1:1000 dilution) for 30 min. The membrane was washed in PBS-Tween 20 four times for 10 min each. The film was exposed to detect and subsequently developed for analysis.

RESULTS

Aerosol Characteristics. The mean aerosol concentrations and SDs were 1.3 ± 0.7 (n = 12), 20.7 ± 10.1 (n = 36), and 481 ± 234 (n = 36) μg isotretinoin/l for the low, mid, and high exposures, respectively. The MMADs (geometric standard deviations) for the low, mid, and high doses were 1.00 (2.08), 1.33 (1.76), and 1.64 (2.61) μm, respectively. [The progression to larger MMADs at the higher aerosol concentrations results from higher relative concentrations of nonvolatiles, which dominate the compositions after the ethanol vehicle partially evaporates; thus, the percentages of nonvolatiles, including α-tocopherol acetate and ascorbyl palmitate stabilizers, were 0.21, 0.3, and 1.2%, leading to minimum droplet sizes (i.e., if all of the ethanol had evaporated) of 0.38, 0.43, and 0.69 μm, respectively. The minimum droplet sizes were calculated by assuming a MMAD of 3 μm for the Pari-LC jet plus nebulizers used in these experiments and using the relationship \( d_{\text{final}} = d_{\text{orig}} \times \frac{1}{3} \), where \( d_{\text{final}} \) is the final diameter, \( d_{\text{orig}} \) is the original diameter, and \( f \) is the mass fraction of solute.]
tumorigenesis in some organs (26) but increases tumorigenesis in the lung (27).

Hyperplastic areas were significantly elevated in the NNK- and BaP-treated mice exposed to mid and high doses of isotretinoin. Total lesions, i.e., hyperplastic areas plus adenomas, were not affected by treatment in the NNK-induced animals but were fewer for the urethane-treated animals at the high isotretinoin dose and for the BaP-treated animals at both the mid and high isotretinoin doses (Table 3).

For the animals receiving low doses of isotretinoin, the numbers of tumors were not affected by treatment at the 95% confidence level, but for both the NNK- and BaP-treated mice, trends in line with those of the mice receiving mid and high doses of isotretinoin were evident for both tumors and hyperplastic areas (Table 3).

For the urethane and BaP treatments, the mice exposed to vehicle had fewer tumors than the cage control animals (Table 3). This phenomenon was observed to an even greater degree in a chemoprevention study in A/J mice with aerosolized budesonide, where the control mice were exposed essentially to air only (28), and possibly is related to the tumorigenesis-inhibiting effect of stress (29), although a contribution from the ethanol vehicle or the antioxidant excipients, ascorbyl palmitate and \( \alpha \)-tocopherol, cannot be ruled out in the studies reported here.

**Discussions**

Despite promising initial clinical reports and considerable basic interest in retinoids as lung cancer chemopreventive agents, there has been surprisingly limited work with these agents in preclinical efficacy models. In this pilot experiment, we used the carcinogen-induced A/J mouse model to begin exploring some simple pharmacology issues. The preliminary nature of this work precludes making definitive conclusions, but a series of observations are supportable.

**Biomarkers: RAR Induction.** Inhaled mid-dose 13-\( \text{cis} \) retinoic acid up-regulated lung tissue RAR\( \alpha \) by 3.9-fold over solvent, RAR\( \beta \) by 3.3-fold, and RAR\( \gamma \) by 3.7-fold, showing that these receptors are useful biomarkers of retinoid activity in this system (Fig. 2). An explanation for the apparent increases in RARs in group 1 relative to group 5, which differed only in that the group 1 animals had received an i.p. injection of urethane 16 weeks prior to sacrifice, is not available and must await further research to determine the reproducibility of the relatively small increases. Similarly, more studies are needed to determine the significance, if any, of the apparent induction of RARs in the group 2 mice exposed to vehicle aerosol.

**DISCUSSION**

- **Fig. 1** Body weights of BaP-treated A/J mice exposed to isotretinoin aerosols. The body weights for the BaP-treated mice are representative and typical of those for all three carcinogen treatments.
  - +, unexposed control; \( \bullet \), vehicle control (daily exposure); \( \bigcirc \), low dose (daily exposure); \( \square \), mid dose (daily exposures first 12 days, then three times weekly); \( \blacktriangle \), high dose (daily exposures first 12 days, then twice weekly); \( * \), 9 of 21 animals sacrificed moribund.

**Table 4** Body weights of carcinogen-treated, isotretinoin-exposed A/J mice near termination of exposures

<table>
<thead>
<tr>
<th>Isotretinoin aerosol level</th>
<th>Urethane-treated mice Day 60</th>
<th>NNK-treated mice Day 102</th>
<th>BaP-treated mice Day 102</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unexposed control</td>
<td>24.7 ± 2.1 (45)</td>
<td>26.6 ± 2.2 (41)</td>
<td>25.6 ± 2.6 (41)</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>20.8 ± 1.2 (21)</td>
<td>22.7 ± 1.0 (21)</td>
<td>22.5 ± 1.4 (21)</td>
</tr>
<tr>
<td>Low dose</td>
<td>21.0 ± 1.2 (21)</td>
<td>22.1 ± 1.5 (21)</td>
<td>21.8 ± 1.2 (21)</td>
</tr>
<tr>
<td>Mid dose</td>
<td>20.7 ± 1.5 (21)</td>
<td>20.3 ± 1.2 (20)( a,b )</td>
<td>20.1 ± 1.8 (20)( a,b )</td>
</tr>
<tr>
<td>High dose</td>
<td>18.9 ± 1.7 (12)( a,c )</td>
<td>17.5 ± 1.2 (14)( a,d )</td>
<td>19.3 ± 1.6 (12)( a,e )</td>
</tr>
</tbody>
</table>

*\( a \) *P < 0.05, different from vehicle control.
*\( b \) One NNK-treated mouse sacrificed moribund in week 13; one BaP-treated mouse found dead in week 4.
*\( c \) Nine mice found dead or sacrificed moribund in week 2.
*\( d \) Five mice found dead or sacrificed moribund in week 2; one in week 5; one in week 7.
*\( e \) Nine mice found dead or sacrificed moribund in week 2.
Inhaled Isotretinoin

Inhaled Isotretinoin. Ethanolic solutions of isotretinoin were aerosolized with particle sizes calculated to provide substantial pulmonary deposition. The ethanol was not removed from the exposure air. The inhaled ethanol, as well as the excipients α-tocopherol and ascorbyl palmitate, may have had an effect on carcinogenesis for the urethane and BaP treatments because the vehicle-exposed animals had fewer tumors than unexposed controls. However, the effect in these experiments—20 and 30% decreased tumor multiplicity for urethane and BaP, respectively—was less than that observed by others (50%) when BaP-treated control mice were exposed to essentially air alone (28). In any case, the addition of isotretinoin to the aerosols produced significant decreases in tumors relative to vehicle-only aerosols.

Lung Tumor Prevention by Inhaled Retinoids. In this study, we looked at three different doses of isotretinoin aerosols inhaled daily. The lowest dose was not significantly effective. The highest dose was associated with lethal toxicity, presumably due to extensive ulceration of the snout and forearms of the mice. The assumption was that this was related to the well-known local toxic effects of retinoids on skin. This apparent local toxic response resolved with a reduction in dose frequency, and significantly fewer lung nodules occurred for all three of the carcinogens with this dosing schedule. In light of the frequent lethal toxicity associated with the high-dose exposures, however, we restricted our focus to the mid-dose exposure as being the relevant drug dose.

With the mid dose, significant toxic signs were not observed other than the weight loss that occurred near the end of the study (Fig. 1). The 3% fatality rate in this cohort would not be unusual in an experiment involving this degree of manipulation of the experimental animals, the absence of deaths in the low-dose and vehicle-exposed groups possibly being a statistical fluke. Even in the vehicle controls, there was a >10% weight difference, relative to unexposed animals, due to the experimental procedure. The stress of forced aerosol inhalation in rodents is expected to be very different from voluntary aerosol inhalation in humans.

Despite the reduction in inhaled dose frequency taken as a precaution against potential local nasal toxicity, the mid dose was still associated with a significant reduction in the number of lung nodules for both of the tobacco-related carcinogens, BaP and NNK. This finding is even more significant when considering the amount of drug that was required to achieve this effect. For example, over most of the study, the mid-dose level, including extrapulmonary dose, was <0.5% of an oral dose used in the previously discussed in vivo experiments (Table 1); based on pulmonary dose alone (Table 2), the dosage was <0.15% during the first 2 weeks and <0.06% during the remainder of the experiment. By all accounts, these comparisons suggest remarkable drug potency for the inhaled aerosol.

The finding that a modest dose of inhaled retinoid is both tolerated and efficacious supports the contention that lung therapy by inhalation is the preferred route of lung delivery for dealing with the airway-confined phase of a disease process, as has been reported with certain agents used to treat pulmonary infections (30) and corticosteroids for cancer prevention (28, 31). An obvious application for the inhalation approach is the use of retinoids as lung cancer chemopreventive agents.

Hyperplasia and Total Lesions: Mode of Action. The preliminary data for the NNK-treated mice suggest that isotretinoin does not eliminate initiated cells but inhibits their progression to the tumor stage: hyperplastic areas inversely correlated with tumors, whereas total lesions, i.e., hyperplastic areas plus adenomas, remained relatively constant (Table 3). A similar increase in hyperplastic areas occurred in the BaP-treated mice, but in this case, total lesions decreased, suggesting that initiated cells were either eliminated or were constrained to microscopic clusters.

BaP and NNK are putative major carcinogens in tobacco smoke (32), and thus are the most relevant of the carcinogens used in this study. The similarities between the dominant molecular lesions caused by BaP and NNK—BaP causes G-C to T-A transitions in the first nucleotide and NNK causes G-C to A-T transitions in the second nucleotide, both in codon 12.
In contrast to the NNK- and BaP-treated animals, tumor multiplicity in the urethane-treated mice was decreased only at the high isotretinoin dose, the meaning of which is obfuscated by associated toxicity, and there was no effect on numbers of hyperplastic areas (Table 3). The total number of lesions was markedly reduced in the high-dose animals, suggesting elimination of initiated cells or restriction of clonal expansion to microscopic lesions. Like NNK and BaP, urethane, an ethylating agent, mutates K-ras, but at codon 61 instead of codon 12 (33). Morphological differences in tumors also occur. The fractions of tumors classified as solid tumors were 78 and 88% for BaP- and NNK-induced tumors, respectively, but only 57% for urethane-induced tumors (34). It is interesting to speculate that these differences contribute to the varied responses to inhaled isotretinoin, but there appears to be no supportive data in the literature.

**Retinoid Toxicity at Efficacious Doses.** Although the high dose with the twice-weekly schedule was only 6% of a nontoxic oral dose (Table 1), it was associated with weight loss toward the end of the study (Fig. 1 and Table 4). For the NNK- and BaP-treated mice, this dose was essentially no more efficacious than the much smaller mid dose (Table 3). Perhaps surprisingly, the mid inhaled dose at only 0.4% of a nontoxic oral dose also caused weight loss in mice exposed for >10 weeks. Examination of the weight data over the duration of the experiment (data from BaP-treated mice in Fig. 1; data from NNK- and urethane-treated mice not shown) confirms the late onset of the weight loss.

There are at least two possible explanations for this finding: (a) the total dose may have been higher than calculated as a result of uptake through the skin of the exposed snout; and (b) local toxicity may have occurred in the respiratory tract. It seems unlikely that sufficient isotretinoin could have been absorbed through the skin to produce systemic toxicity, nor would such a conclusion be supported by numerous inhalation studies in mice with aerosols of other compounds. This leaves local toxicity as a possible explanation. Microscopic examination of tissues for pathological changes was neither planned nor carried out for this pilot efficacy study; however, gross examination of the lungs revealed no differences between control and treated lungs except for the differences in numbers of tumors and hyperplastic areas. Given that the pulmonary dose is calculated to be only 13% of the total deposited dose and would be distributed over ~640 cm² (35), pulmonary toxicity seems unlikely.

In contrast to the large surface of the lung, the upper respiratory tract, mostly nasal mucosa, has a surface area of only ~3 cm² (36) but receives ~87% of the deposited dose. Coupling this high dose with the ease with which rodents develop debilitating nasal lesions (37, 38), we suggest that an explanation for the weight loss in the mid-dose mice is local nasal toxicity, which developed to significant levels after ~10 weeks of exposure.

Only toxicology studies with histopathology included will provide definitive explanations for the phenomenon of weight loss at these low doses, but if our suggestion that upper respiratory tract toxicity is to blame is correct, the observation is probably not relevant to the safety of inhaled retinoids in people. For pulmonary toxicology studies except for neoplasia, nasal toxicity in rodents, which are obligate nose breathers, from inhalants is usually not considered relevant for evaluating possible effects in humans unless human exposure will include the nasal cavity. For example, local nasal toxicity would not be a concern with a chemopreventive agent administered by oral inhalation because this route of administration skips drug transit across the nasal cavity. Moreover, the dose-response curve for efficacy appears to have already plateaued at the mid dose (Table 3), suggesting that the dose could be lowered to a point between the low dose and the mid dose without sacrificing efficacy.

**Limitations of the A/J Mouse Model: The Possible Role of Inflammation in Lung Cancer.** A special limitation of mouse inhalation models is that with the nature of the drug delivery system, a major fraction of the total administered drug will deposit on the snout and in the upper respiratory tract. Without delivering drug via a tracheotomy, there is no other alternative. Therefore, an artifact of this model is inefficient drug delivery to the deep lung. In humans, where much more efficient pulmonary drug delivery devices exist, the fraction of the drug that is impacted in and around the snout in the mouse would be expected to travel directly into the pulmonary airway. This improved drug delivery efficiency would greatly reduce the potential for local toxicity.

For some drugs, the high extrapulmonary deposition in the mouse model might confound interpretations regarding the effectiveness of the pulmonary route of drug delivery. In the case of isotretinoin, we suggest that the extrapulmonary-deposited drug probably is not germane because it would either be swallowed or absorbed into the blood stream in much lower amounts than ineffective oral doses (Table 1) and so is unlikely to have contributed significantly to efficacy.

We used an animal model for evaluation of efficacy. Animal models for human lung cancer are widely accepted (10, 11), but like all preclinical models, the A/J mouse model is imperfect. With the A/J model, mice treated with complete carcinogens do not develop lung inflammation and the attendant rapid cell proliferation that is common in human lung disease. The contribution of inflammation to aerodigestive carcinization is becoming more evident, and this may be, in part, how retinoids effect their chemopreventive benefit in humans.

A connection between the inflammation-associated enzyme COX-2 and retinoid pathways is suggested by the fact that the Ras/extracellular signal-regulated kinase signaling pathway appears to play a role in the regulation of COX-2 expression. Human non-small cell lung cancer cell lines with mutations in K-ras have high expression levels of COX-2, and inhibition of ras activity in these cell lines decreases COX-2 expression (39). Rat intestinal epithelial cells and fibroblasts transfected with H-ras overexpress COX-2, whereas inhibitors of extracellular signal-regulated kinase ameliorate this response (40). We and others have found COX activity to be potentially significant in aerodigestive cancers (41–43). A high percentage of murine and human lung adenocarcinomas have a mutated ras gene and a constitutively activated ras signaling pathway (13), which may explain the high levels of COX-2 seen in some lung tumors. RARβ is known to interfere with the ras signaling pathway by inhibiting the function of the activator protein transcription...
factor (44). An expected result of this interference would be the down-regulation of COX-2 expression, which may play a role in the decreased tumorigenesis seen in the A/J lung cancer model following isotretinoin inhalation. Such an effect of RARs on COX-2 expression is supported by published data showing that retinoids inhibit the epidermal growth factor (i.e., ras)-induced transcription of COX-2 in human oral squamous carcinoma cells (45).

Through time, the development of in vivo models that more closely mirror the actual process of carcinogenesis in humans would be highly desirable. For the pilot evaluations discussed here, we believe the A/J mouse model is adequate as long as its shortcomings are acknowledged. In future experiments, the local aerosol drug dose in and on the snout can be reduced through modifications of the exposure system to prevent facial exposure and by reducing the particle diameter to ~0.3 μm, which will increase the pulmonary-to-total dose ratio to ~64% (24).

**Induction of RARs.** RARs were investigated as biomarkers because their genes contain retinoic acid response elements and as such are likely to be up-regulated soon after exposure to retinoids, i.e., they are first-order dependence genes (46). The induction of all three RARs was at least 3-fold in lungs exposed to mid levels of isotretinoin. Only the urethane-treated mice were examined in this pilot study, but these probably represent the other treatment groups for this determination because all mice were exposed to the same aerosols. The induction of the RARs in the mid-dose mice correlated with efficacy in the BaP- and NNK-treated mice and may not only provide biomarkers for exposure but may also be a part of the mechanism for the efficacy of inhaled isotretinoin. The up-regulation of lung RARs by inhaled isotretinoin occurs across species, as reported in a companion study, which also indicates that oral administration induces liver, but not lung RARs, and that administration by inhalation induces only lung, and not liver receptors.

**Implications of Improving the Therapeutic Index of Retinoid Administration.** The clinical trials to unequivocally establish the chemopreventive benefit of oral isotretinoin are likely to be completed in the near future. Even if positive, however, long-term compliance is expected to be a major issue because of the significant frequency of debilitating side effects. Even if changing the route of administration of retinoids only decreased the side effect profile, this would make the drug much more interesting to contemplate for broad clinical utility. Moreover, because the role of retinoids in maintaining optimal bronchial epithelial differentiation has been extensively studied, other general beneficial effects of retinoic acids on epithelia have been well documented. For example, in a study using a rodent model of chronic obstructive pulmonary disease, there was a suggestion that retinoids can reverse parenchymal lung injuries associated with compromised respiratory function (47). Indeed, if there is a causal relationship, this benefit may contribute to the cancer-preventive effects because individuals suffering from chronic obstructive pulmonary disease and other smoking-related diseases are at increased risk for developing lung cancer (48).

**Chemoprevention of Lung Cancer in Smokers.** Although the role of retinoid biology is thought to be pivotal in the process of tobacco-induced carcinogenesis, attempts to use retinoids as lung cancer chemopreventive agents have been problematic as shown by clinical trials (49–51). In two of three large β-carotene studies, there were a greater number of lung cancers occurring in smokers on the β-carotene arm. In a more recent randomized control trial of 13-cis retinoic acid in stage I resected non-small cell lung cancer, during the first evaluation of the trial, there was a question about the time to disease recurrence in smokers receiving active drug (52). Although the trial was already completed in regard to accrual, the smokers still on retinoid were instructed to stop treatment until the validity of the correlation was sorted out. The speculation of smokers being harmed by efforts to chemoprevent lung cancer is conceptually plausible. The presence of ongoing carcinogenic damage in the setting of the airway of a smoker receiving drug involves complex scenarios in which adverse outcomes are possible. Clearly, this same approach in former smokers represents a more favorable opportunity to document an objective benefit of chemoprevention. In light of this situation, perhaps the most responsible way to proceed is to carefully study the benefit of aerosolized chemoprevention in parallel cohorts based on smoking status. A clinical trial that formally evaluates the outcomes of aerosolized retinoid chemoprevention as a function of smoking status is needed to address this important issue.

In this preliminary analysis of the pulmonary delivery of isotretinoin by inhalation, there was evidence of efficacy at weekly pulmonary doses as low as 0.25 mg/kg and suggested efficacy at doses as low as 0.04 mg/kg in reducing the pulmonary carcinogenicity of the tobacco carcinogens NNK and BaP in A/J mice. Because pulmonary drug delivery deposits drug directly on the tumor compartment, efficacy can be achieved at low doses: mid and low weekly pulmonary doses were <2 and <0.3%, respectively, of the highest recommended weekly oral dose of isotretinoin for acne treatment (Accutane 55; Roche). The results reported here, however, are all of the more encouraging because they probably were produced by the <10% of the inhaled aerosol that deposited in the lung as the extrapolummary dose was probably too low to have a systemic effect. This suggests that an improved therapeutic index can be achieved in humans by more selectively delivering retinoid chemopreventive agents to deep lung tissue using aerosols. Further work with this approach, both preclinically and in the clinic, is justified to validate the true benefit of this important new chemoprevention delivery approach.

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