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Inhaled aerosolization of all-*trans*-retinoic acid for targeted pulmonary delivery

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Abstract Retinoids have shown promising activity for both cancer chemoprevention and as a treatment for emphysema. However, chronic oral administration of these drugs is limited by systemic side effects, including hepatic dysfunction, skeletal malformations, hyperlipidemia, hypercalcemia, and other reactions. In order to improve the pulmonary targeting of this potentially useful therapy, we developed a system for aerosolization of retinoids that substantially increased their local bioavailability. We compared the biodistribution and pharmacokinetics of an inhaled formulation of all-*trans*-retinoic acid (all-*trans*-RA), which was packaged in a metered dose inhaler, following both intratracheal (IT) and intravenous (IV) administration in male Sprague-Dawley rats. After drug administration, anesthetized animals were killed at 5 min, and at 1, 2, 4, 6 and 24 h. Plasma and emulsified samples of liver and lung tissues were dissected, extracted, and frozen prior to measurement of all-*trans*-RA concentration by high-performance liquid chromatography (HPLC). Aerosolization and IT injection of all-*trans*-RA resulted in a significantly longer pulmonary half-life of the drug (both 5–17 h), lower peak serum concentrations (aerosol 71 ± 31 ng/ml, IT 68 ± 50 ng/ml), and lower liver levels (aerosol 111 ± 28 ng/g, IT 753 ± 350 ng/g) than the same dose administered IV (2 h, 838 ± 56 ng/ml, 4258 ± 1006 ng/g, respectively; $P < 0.05$ for each comparison). Histologic examination of lungs and trachea showed no focal irritation attributable to the drug after single-dose administration. These results suggest that aerosolization of retinoids may offer a practical alternative to systemic oral administration for chemoprevention trials or treatment of lung diseases. This method may substantially increase the therapeutic index of these compounds by reducing systemic complications associated with long-term dosing.

Key words All-*trans*-retinoic acid · Chemoprevention · Inhalers

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Introduction

Retinoids are derivatives of vitamin A that are essential for normal differentiation of epithelial cells. In rodents, vitamin A deficiency induces epithelial dysplasia in the tracheobronchial tree, whereas in humans, exogenous treatment with retinoids induces terminal differentiation of certain premalignant lesions of the skin [1], oral mucosa [2], and uterine cervix [3]. These agents have produced striking therapeutic results in patients with acute promyelocytic leukemia [4], where their use has more than doubled the proportion of patients cured of this highly lethal disease [5].

Preliminary trials have shown that retinoids may reduce the incidence of second primary tumors in patients who have received initial therapy for cancers of the head and neck [6] and lung who are at high risk for recurrence due to chronic tobacco exposure [7, 8]. Moreover, new evidence suggests that all-*trans*-retinoic acid (all-*trans*-RA) may induce the proliferation of alveolar cells in lungs that have been damaged by fibrosis [9]. This

surprising observation suggests that retinoids may have important utility for the treatment of patients with pulmonary emphysema.

Chronic treatment with retinoids is associated with a number of side effects, several of which are serious. These reactions include hypertriglyceridemia (possibly leading to accelerated atherosclerosis and pancreatitis), hepatic failure, skeletal pain and malformation, mucositis, hypercalcemia, and birth defects. Indeed, such complications have been associated with high dropout rates or dose reductions in clinical studies [10, 11, 12, 13, 14, 15].

Several potential strategies for mitigating the toxicity of these agents have been considered, including "drug holidays", reductions in dosage, and development of naturally occurring or synthetic ligands that bind specific nuclear retinoid receptors [16]. However, none of these strategies has yet yielded a substantial increase in therapeutic index.

For many drugs, organ-specific targeting, especially by the inhaled route, has been an effective means of drug delivery. In addition to significantly increasing local drug concentrations at the desired site, this route avoids the "first pass" effect that occurs with transport through the liver. Inhalation therapy has proved especially useful for the treatment of lung diseases, including asthma, cystic fibrosis, *P. carinii* prophylaxis, and other conditions [17]. Given the systemic toxicity of retinoids, plus the requirement for protracted administration for cancer chemoprevention and emphysema treatment, we developed an aerosolized delivery system that enabled direct pulmonary delivery of all-*trans*-RA, and we evaluated the comparative pharmacokinetics and bioavailability of this delivery system in a rodent model.

Methods

Stock drug preparation

For the intratracheal (IT) and intravenous (IV) studies, all-*trans*-RA was solubilized in 20% ethanol and 10% Tween 20 with 1 m*M* ammonium hydroxide for a final concentration of 1 mg all-*trans*-RA per milliliter of solution. A mixture of 20% ethanol and 10% Tween 20 served as control.

Inhaled drug preparation

For the inhaled aerosol studies, all-*trans*-RA was solubilized in tetramethyl ammonium hydroxide and mixed with hydrofluorocarbon (HFC) 123 to a concentration of 5 mg/ml. This solution was then combined with hydrofluoroalkane 134a as propellant, for a final concentration of 1.5 mg per 1.475 g of propellant solution. Of this final solution, 21 g was then packaged in a multidose aerosol canister which dispensed 77.5 mg of product per dose. (Thus, each puff was projected to deliver approximately 80 μg of all-*trans*-RA.) An identical canister containing propellant mixture without drug was used as a control. The canisters were kindly provided by Sciarra Laboratories (Hicksville, NY).

Pharmacokinetics

Animals in the inhaled drug group were anesthetized and intubated endotracheally under direct vision using a 20 gauge angiocatheter over a guidewire, as described previously [18]. An apparatus for the delivery of aerosol through an angiocatheter was fashioned by modifying a plastic metered-dose canister holder and welding it to a standard IV catheter hub (Fig. 1). This apparatus was connected to the endotracheal tube of the anesthetized animal, and three doses of aerosolized solution (240 μg all-*trans*-RA or control) were delivered coincident with the animal's spontaneous inspiration. Animals in the IT group were intubated as above, and 250 μl of stock all-*trans*-RA solution (250 μg) was injected through the angiocatheter. Animals in the IV group underwent surgical exposure and isolation of the right external jugular vein. Using a 1 ml syringe fitted with a 26-gauge needle, 250 μl of all-*trans*-RA (250 μg) or control solution was injected as a single IV bolus. Upon completion, blood was aspirated to verify needle placement. Within each group, four to six animals were killed at 5 min, and at 1, 2, 4, 6 and 24 h, after receiving the all-*trans*-RA or control preparations. A group of five animals that had not received any inhaled agent were killed to serve as controls. Plasma was separated from heparinized whole blood, and lungs and liver were dissected from all animals. All samples were then frozen at −20 °C until analyzed. In addition, one set of lungs from each treatment group at each time-point was placed in formalin and sent for histologic examination to evaluate local toxicity.

Animal care procedures

All procedures were performed in accordance with NIH guidelines for proper care and use of laboratory animals, and followed a protocol that was approved in advance by this center's Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Charles River Laboratories) received pentobarbital at a dose of 50 mg/kg intraperitoneally as anesthesia for all experiments.

Verification and calculation of the effective delivered dose

The actual amount of drug delivered to the lungs (defined as the "effective dose") was calculated by delivering ten doses through the aerosol delivery apparatus into a flask that contained 100 ml isopropanol. This procedure was also repeated with an angiocatheter fitted to the apparatus. The catheter was subsequently rinsed with

Fig. 1 Prototype of metered dose inhaler with modified adapter for use with the angiocatheters used for endotracheal intubation of the rat

10 ml isopropanol, which was also collected. The three solutions were then assayed for all-*trans*-RA concentrations in order to determine the amount of drug delivered through the apparatus, as well as any residual drug that was bound to the angiocatheter. We defined the area under the concentration-time curve (AUC) for animals that received IT all-*trans*-RA as 100% effective dose delivery to the lung. The AUC of the inhaled aerosolized drug was divided by the IT AUC in order to determine the percent effective dose.

Analytical procedures

All tissues were weighed and mechanically homogenized in 100% isopropanol. The samples were centrifuged and the supernatants were saved for analysis. Plasma was deproteinized in an equal volume of isopropanol and centrifuged to obtain supernatant. Sample processing and high-performance liquid chromatography (HPLC) analysis were performed as reported previously [19] with a minor modification. Solid-phase extraction was performed off-line using a C2 Bond Elute cartridge, and the eluent was evaporated to dryness in a vacuum. Samples were resuspended in buffer (80% acetonitrile/20% ammonium acetate with 0.2% acetate) and were analyzed using a Spherisorb ODS-2 column and isocratic elution. The retention time for all-*trans*-RA was 23–24 min (Fig. 2).

Pharmacokinetic calculations and statistical analysis

Pharmacokinetic calculations were performed using the WinNonLin software package (Scientific Consulting, Apex, N.C.). Pharmacokinetic results were based on a noncompartmental model. AUCs in plasma, liver, or lungs were calculated according to the log-linear trapezoidal method. Analysis of variance (ANOVA) was used to compare means between groups at various time-points. Data are reported as means ± SEM.

Results

Pulmonary delivery of all-*trans*-RA

In animals that received the metered dose inhaler, lung concentrations of all-*trans*-RA were highest at 5 min (99 ± 44 ng/g tissue), followed by a slow decrease over the next 24 h (Fig. 3). The calculated tissue half-life of the aerosolized drug in lung was 5.4 h. Animals that received IT injections showed peak lung levels at 1 h (5767 ± 419 ng/g), which were maintained for approximately 6 h. The lung tissue half-life ($T_{1/2}$) was 17.7 h by the IT route. Peak lung levels by the IV route were seen at 5 min (6440 ± 1865 ng/g) and disappeared with a half-life of 1.9 h. The lung levels by the inhaled route were significantly lower than by the IT route at each time-point ($P < 0.02$); however, the difference from the IV route was only significant before the 2-hour post-dosing time-point. The lung AUC, an indication of total drug delivery to an organ over time, for the IT route was twice that for the IV route and 100 times that for the aerosolized route (Table 1).

Plasma levels of all-*trans*-RA

Animals receiving aerosolized drug displayed peak plasma levels of 71 ± 31 ng/ml 1 h after dosing, which was not significantly different from that following IT administration at 1 h (68 ± 44 ng/ml; Fig. 4). By contrast, animals in the IV group displayed a characteristic plasma curve with a peak at 5 min (838 ± 56 ng/ml) and a rapid decay ($T_{1/2}$ 0.4 h). Differences between the IV and the other groups were significant up to 4 h ($P < 0.05$).

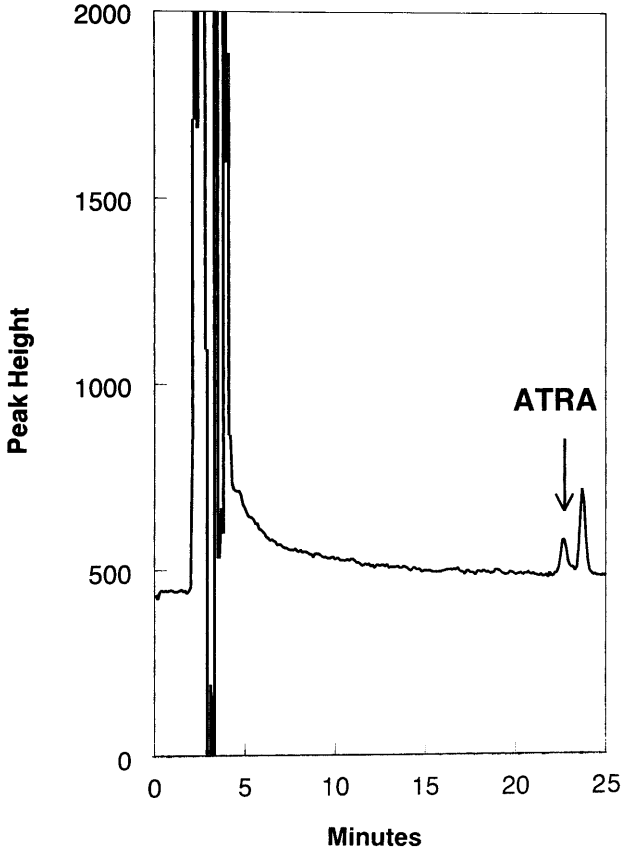

Fig. 2 Representative chromatogram showing all-*trans*-RA (*ATRA*) peak at 22–23 min

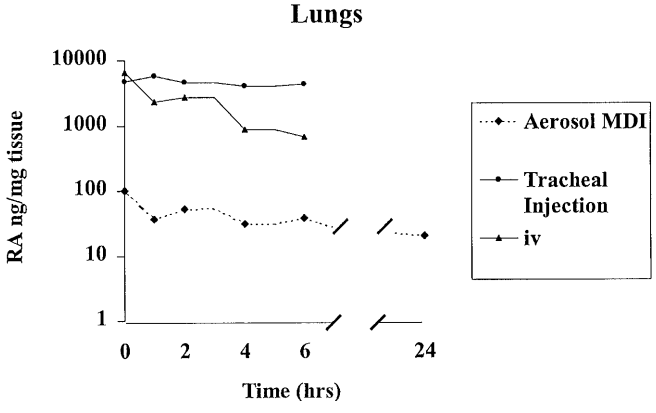

Fig. 3 Log of mean lung levels of all-*trans*-RA following administration of a 240-μg dose by inhalation (three to five doses per time-point), 250 μg IV (five doses per time-point), or 250 μg IT injection (three doses per time-point)

Table 1 Organ AUC (ng · h/g or /ml) and half-life (h) of all-*trans*-RA after inhaled, IT or IV dosing

	Lungs		Plasma		Liver	
	AUC	$T_{1/2}$	AUC	$T_{1/2}$	AUC	$T_{1/2}$
Intratracheal	26,972	17.7	191	1.9	2532	22
Intravenous	11,910	1.9	1126	0.4	4724	0.8
Inhaled	262	5.4	171	3.0	2307	–[a]

[a] Unable to calculate $T_{1/2}$ without decay curve

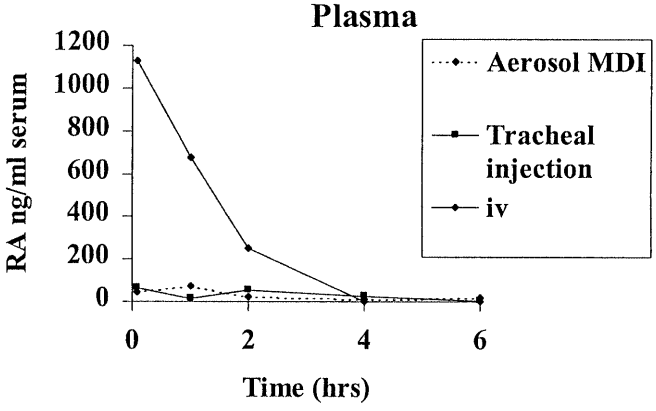

Fig. 4 Mean plasma levels of all-*trans*-RA following administration of a 240-μg dose by inhalation (three to five doses per time-point), 250 μg IV (five doses per time-point), or 250 μg IT injection (three doses per time-point)

Hepatic levels of all-*trans*-RA

Animals in the inhaled drug group had peak liver levels of 112 ± 28 ng/g 2 h after dosing, whereas IT injection produced peak liver levels of 753 ± 350 ng/g at 5 min; (Fig. 5). Both groups showed a slow decrease over 24 h. In contrast, IV administration resulted in peak levels in liver at 5 min (4258 ± 1006 ng/g), which had rapidly decreased by 4 h ($T_{1/2}$ 1 h).

Effective dose

Although projected to deliver 80 μg, we found that the actual dose delivered from each metered-dose canister was 120 μg. However, the use of an angiocatheter as an endotracheal tube in this model markedly decreased the amount of drug delivered to 49 μg (41%), and an additional 6 μg of drug was bound to the tube itself. After dividing the AUC after IT injection (26,972 ng · h/g) by the AUC for the inhaled aerosol (262 ng · h/g), a calculated effective dose of 1% was obtained, which indicates that approximately 40% of the drug was lost in the oropharynx (or outside the animal) in this model.

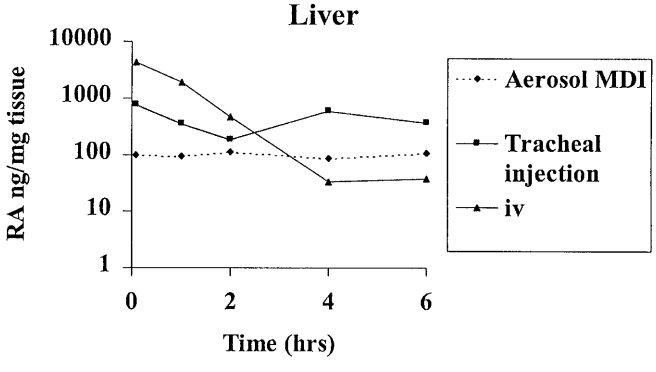

Fig. 5 Mean hepatic levels of all-*trans*-RA following administration of a 240-μg dose by inhalation (three to five doses per time-point), 250 μg IV (five doses per time-point), or 250 μg IT injection (three doses per time-point)

Toxicity

There were no deaths after drug treatment, and all animals that recovered from anesthetic exhibited normal behavior until the time of killing. Animals not recovering from anesthesia were necropsied to determine the cause of death. In the majority of animals which died in early experiments using the inhaler alone, the cause of death appeared related to barotrauma from the relatively high-pressure device.

Histology

Paraffin sections of treated and control lungs obtained at each time-point were examined by light microscopy. The lung parenchyma demonstrated no pathologic changes. Two specimens (i.e. from the 24-h inhaled drug and the 4-h inhaled control) demonstrated a focal tracheitis with some foreign material noted at the site of the inflammation. This injury is consistent with trauma induced by intubation or freeze injury from the low temperature of the propellant solution.

Discussion

Chemoprevention of aerodigestive tract cancers is based on the principle of field cancerization [20]. Patients who have been treated for head and neck cancer have an increased incidence of second primary malignancies owing to extensive tobacco exposure [21], and this observation has led to the evaluation of the retinoids for secondary prevention of such tumors [6]. Results from preliminary trials have suggested that this therapy may reduce the incidence of second primary tumors [22].

Because of the lack of biomarkers in aerodigestive tract cancers, the actual incidence of new tumors must be measured, which requires both protracted therapy and long-term follow up. While results of early chemo-

prevention trials are encouraging, these results reflect relatively short-term therapy (i.e. <2 years). Current chemoprevention trials with retinoids are solely confined to orally administered drugs [2, 6, 7], a route of administration that exposes the drug to first-pass metabolism in the liver. Moreover, continuous daily treatment with all-*trans*-RA is associated with a progressive reduction in plasma drug levels [23, 24], which may further reduce clinical efficacy.

Clinical use of oral retinoids is associated with high incidences of adverse effects, including cheilitis, skin reactions, headache, hyperlipidemia, and hepatic dysfunction [10, 11, 12, 13, 14, 15]. Moreover, toxic reactions have been responsible for dose reduction or cessation of therapy in up to 42% of patients in some studies [10]. The use of retinoids for the management of pulmonary emphysema will also undoubtedly require chronic administration, and the avoidance of toxicity in these patients is essential to achieve maximal compliance. While the negative consequences of long-term retinoid therapy in these patients are unknown, they are probably quite important given the size of the target population.

The advantages of inhaled drug delivery are well characterized. Site-specific drug delivery is readily accomplished, and the portability of multidose canisters, their ease of use, and reduced adverse effects should improve compliance. Moreover, the avoidance of elevated serum and liver drug levels should further enable long-term therapy for cancer chemoprevention in high risk (but asymptomatic) persons, as well as individuals with fibrotic lung disease.

This study showed that local delivery of all-*trans*-RA to the lung results in low systemic levels and a long half-life in the target tissue. However, relative to direct IT instillation, the effective delivery of all-*trans*-RA to the lung by the aerosolized route in this model was only 1%. This percent-effective delivery is low, but is not strikingly dissimilar from that of inhaled steroids and bronchodilators, which yield an effective delivery of 6–10% [17]. Furthermore, these drugs are delivered at a fraction of the systemic dose required for the same effect. The amount of all-*trans*-RA required in the lung for adequate chemoprevention is unknown.

There are numerous limitations to the rat model we employed. First, while rat lung volume ranges from 2 to 5 ml, the volume of air delivered by each insufflation is 15 to 30 ml, which considerably exceeds organ capacity. Indeed, a number of early animals tested died from barotrauma induced by the insufflation. Second, in surviving animals, a large amount of aerosol was found to have escaped back through the nose and mouth (so called ''blowback''), further reducing lung delivery. Third, in humans, the use of the metered-dose inhaler is timed with inspiration, so the aerosol is drawn into the lungs by negative pressure. In the rat model, the aerosol was delivered by positive pressure that may have blown drug out of the lungs or even across alveolar septa. Thus, while highly useful for the screening and feasibility tests we have described, the model has obvious limitations compared with testing in larger animals or humans.

Nonetheless, the pharmacokinetics of this model show that inhaled doses of all-*trans*-RA have a half-life in the lung in the range 5–17 h, which would enable dosing up to two to three times per day. While liver toxicity was not directly measured, at equidose levels both IT and inhaled all-*trans*-RA yielded significantly lower liver levels compared with IV administration. Finally, plasma drug levels were not significantly elevated by either IT or inhaled dosing. Since systemic retinoid toxicity is both dose- and concentration-dependent, inhaled retinoids should produce a markedly lower incidence of systemic toxicity relative to oral ingestion.

Despite limitations of this model, this study shows that inhaled therapy with aerosolized retinoids is feasible, and that aerosolization may considerably increase both the therapeutic index and utility of these compounds for both cancer chemoprevention and treatment for emphysema.

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