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Uptake of all-*trans* retinoic acid–containing aerosol by inhalation to lungs in a guinea pig model system—A pilot study

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Abstract

Systemic therapies with retinoic acid (RA) can result in toxic side effects without yielding biologically effective levels in target tissues such as lung. The authors adapted a PARI LC Star nebulizer to create a tubular system for short-term inhalation treatment of guinea pigs using a water-miscible formulation of all-*trans* RA (ATRA) or vehicle. Based on the initial average weight, animals received an estimated average ATRA doses of either 0.32 mg·kg⁻¹ (low dose, 1.4 mM), or 0.62 mg·kg⁻¹ (medium dose, 2.8 mM), or 1.26 mg·kg⁻¹ (high dose, 5.6 mM) 20 minutes per day for 6 consecutive days. This system led to a rise of ATRA levels in lung, but not liver or plasma. Cellular lung levels of retinol, retinyl palmitate, and retinyl stearate also appeared to be unaffected (245.6 ± 10.7, 47.4 ± 3.4, and 132.8 ± 7.7 ng·g⁻¹ wet weight, respectively). The application of this aerosolized ATRA also induced a dose-dependent protein expression of the cellular retinol-binding protein 1 (CRBP-1) in lung, without apparent harmful side effects.

Keywords

aerosol; CRBP-1; guinea pig; inhalation; lung; retinoic acid; therapy

Biologically active derivatives of vitamin A, most notably retinoic acid (RA) isomers, exert a wide variety of effects on development and cellular differentiation [1]. The actions of RA are mediated by nuclear retinoid receptors, RARs and RXRs [2], each encoded by three genes (RAR α , β , γ and RXR α , β , γ) [3]. Vitamin A and its derivatives also show therapeutic activity toward several cancer and skin disorders [4].

The major problem with the use of retinoids is their toxicity. ATRA (tretinoin) causes headache, nausea, vomiting, bleeding, irregular heartbeat, hearing loss, liver failure, plus teratogenic effects in pregnant women [5].

Massaro and Massaro [6] reported in their work that RA treatment reverses anatomical characteristics and increases lung volume of elastase-induced emphysema in Sprague-Dawley rats. Although demonstrating a beneficial effect, animals received the RA treatment via intraperitoneal administration.

Two recent human trials investigated the effects of orally applied 13-*cis* retinoic acid [7] in patients with stage I non-small-cell lung cancer and 9-*cis* RA [8] used in former smokers.

Both studies reported significant toxic side effects. Thus, the ratio of efficacy to toxicity, termed the “therapeutic ratio,” became a critical consideration.

Inhalation therapy seems a more hopeful approach. Two previous reports described the application of ethanolic 13-*cis* RA formulations in mice [9] and rats [10] using nose-only exposure. In contrast, when Wang et al. [10] gave dietary pharmacological doses of RA, it failed to induce RARs in mouse lung (in contrast to liver RARs), again supporting an inhalation rather than oral approach. In addition, systemically administered retinoic acid is only poorly available to target tissues, because of retinoid interaction with blood albumins [11].

Using a PARI-based inhalation device, in this pilot study we exposed guinea pigs to a 100% water-soluble ATRA nebulizing solution. We evaluated the uptake of inhaled ATRA in guinea pig lungs by high-performance liquid chromatography (HPLC) analysis. Furthermore, we also analyzed the effects of inhaled ATRA on a direct RA-inducible biomarker, the cellular retinol-binding protein (CRBP-1), which has been shown to contain a RA-responsive element in its promotor [12,13].

CRBP-1 plays a major role in the cellular uptake of retinol from the blood into the cells via a receptor-bound mechanism (STRA6) [14] and in the generation of intracellular vitamin A storage via lecithin:retinol acyltransferase (LRAT) [15]. Furthermore, CRBP-1 acts to chaperone retinol and retinal to retinoid-metabolizing enzymes [16].

Materials and Methods

Reagents and apparatus

HPLC-grade acetonitrile, methanol, hexane, tetrahydrofuran (THF), acetic acid (glacial), and ethanol were from Fisher Scientific (Pittsburgh, PA). Cremophor EL and standards (13-*cis*-, 9-*cis*-, all-*trans* retinoic acid, retinol, and retinyl acetate) were from Sigma-Aldrich (St. Louis, MO); standard solutions were prepared and stored at -80°C under low-light conditions.

We fitted a PARI LC Star nebulizer (PARI Respiratory Equipment, Midlothian, VA) to a tubular system, allowing simultaneous inhalation treatment of 6 guinea pigs (Figure 1), with mouths and noses covered by outlet masks. The formation of the aerosol was driven by a PARI PRONEB Ultra compressor. To ensure a constant flow of aerosol, a slight negative pressure was applied to the chamber's outlet. A glass fiber filter (No. 24 Glass, size 4.7 cm; Schleicher & Schuell, Riviera Beach, FL), was placed directly after this outlet, trapped uninhaled aerosol. The inhalation manifold was designed for mouth and nose uptake.

Twelve animals were divided into 4 groups. Group I received low dose (1.39 mM) of aerosolic ATRA, group II received the medium dose (2.78 mM), and group III received high dose (5.55 mM) of aerosolic ATRA. Group IV served as a control that received vehicle only. They received 3 mL of RA aerosol (or vehicle) of these concentrations for 20 min/day for 6 days. On the 6th day, after the last ATRA exposure, the animals were sacrificed at different time points (50, 100, and 150 minutes).

Aerosol preparation and HPLC analyses

Final aerosol component concentrations are shown in Table 1. Cremophor EL, used in this study, is a nonionic solubilizer and emulsifier [17]; the major component is glycerol–polyethylene glycol ricinoleate. Butylated hydroxytoluene (BHT) was added as an antioxidant. To prepare the solutions, Cremophor EL was mixed with ATRA and BHT heated to 60°C . Water and phosphate-buffered saline (PBS) (10×) at 60°C , were slowly

added. After cooling to room temperature, water was added to the desired final volume. ATRA concentrations were determined spectroscopically. Preparations were carried out under dim yellow light, and aliquots frozen at -80°C . HPLC-analyses and sample extractions were performed as reported earlier [18]. According to the manufacturer PARI, the mass median diameter for a typical aerosol is 2.9 micron in a PARI LC Star nebulizer driven by a PRONEB Ultra Compressor.

Dose estimation

After each inhalation procedure, the remaining RA aerosol preparation was collected from the PARI unit and its volume was assessed. Filters at the outlet of the tubular inhalation system were collected and stored at -80°C until analysis. In order to find out the RA concentrations, filters were extracted with 5 mL n-hexane. The extract and the RA aerosol leftovers were diluted and analyzed spectroscopically at a wavelength of $\lambda = 350\text{ nm}$ for their RA content. Doses were estimated based on the calculation, from the initial RA amount of the aerosol preparation subtracting the RA amount left in the PARI unit minus the RA trapped in the outlet filter (i.e., the amount of the RA aerosol that was not inhaled).

Animal housing and tissue preparation

Twelve 5- to 6-week-old male guinea pigs (Hartley Strain, HsdPoc:DH, *Cavia porcellus*) obtained from Harlan (Indianapolis, IN) received diet and drinking water ad libitum, were housed under standard conditions, maintained on a guinea pig diet (no. 7006; Harlan Teklad, Madison, WI) and sacrificed by decapitation. Animals weighted an average of $501.8 \pm 8.5\text{ g}$ at arrival and $588.7 \pm 13.8\text{ g}$ at time of sacrifice on the 6th day. Tissues were removed, flushed with ice-cold phosphate-buffered saline (PBS), flash-frozen in liquid nitrogen, and stored at -80°C away from light. For HPLC analysis, an average of $0.36 \pm 0.02\text{ g}$ per lung sample was taken from frozen tissue (which represents an average of 11.2% of the total lung weight per animal), if possible from the distal regions of the lung. Trachea was removed and bronchial branches were avoided to be included in the samples.

The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Meharry Medical College.

Western blot analysis

Tissue was homogenized with a Brinkman polytron (setting 6–7, 30 s) in 5 volumes of ice-cold PBS, pH 7.2, containing a Protease Inhibitor Cocktail (0.01%; Sigma-Aldrich, St. Louis, MO). Protein was determined by the Lowry method [19]. Equal amounts of lysates ($40\text{ }\mu\text{g}$ protein) were electrophoresed on 4% to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 2% dry milk in 100 mM Tris-buffered saline containing 0.1% Tween (TBST), incubated in primary antibody (affinity purified rabbit polyclonal antibodies against CRBP-1 [20] overnight at 4°C , washed with TBST, incubated with an anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA), washed, and complexes detected using a chemiluminescence kit (Perkin-Elmer, Boston, MA). For normalization, blots were reprobed with anti β -actin antibody (Santa Cruz Biotechnologies). A gel documentation system (Bio-Rad, Hercules, CA) with Quantity One analysis software was used to quantify bands.

Statistical evaluations

Values for biochemical variables are given as mean $\pm$ standard error (SEM). Statistical analysis was performed using GraphPad prism Version 5.0 (GraphPad Software, San Diego, CA).

Results

Description of the inhalation apparatus

To test our aerosols, we adapted a PARI LC Star nebulizer to a tubular system (3.8 cm diameter $\times$ 50 cm length) allowing for the simultaneous treatment of up to 6 guinea pigs (Figure 1). The inhalation manifold was designed for mouth and nose uptake. The setup doesn't distinguish between those 2 routes, therefore we cannot rule out an additional uptake of retinoic acid in the nasal mucosa. Because we did not detect any increase in retinoic acid and isomers in plasma, we assume that no considerable amount was swallowed by the animals.

At the outlet of the system, a filter holder with filter was attached in order to trap aerosol not inhaled by the guinea pigs.

Characterization of the aerosol and dose delivery estimation

An aliquot of the ATRA aerosol was diluted in ethanol and analyzed for RA isomers and oxidized products. Figure 2A is a chromatogram of retinoid standards, and Figure 2B is a chromatogram of freshly prepared ATRA aerosol (99.35% ATRA). Storage (16 months) of the ATRA aerosol at -80°C caused formation of primarily the 13-*cis* RA isomer (3.06% versus original concentration of 0.13%; Figure 2C), reducing ATRA to 96.22%. Three additional peaks ($t_{\text{r}5} = 4.11$ min; $t_{\text{r}6} = 5.18$ min; $t_{\text{r}7} = 7.71$ min) (Figure 2D) were observed. The observed light absorptions [21] point to oxidation products of RA, in particular to 4-oxo RA (5: $\lambda_{\text{max}}(\text{hexane}) = 350$ nm), 5,6-epoxy RA (6: $\lambda_{\text{max}}(\text{hexane}) = 338$ nm), and di-*cis* RA isomer (either 9,13-di-*cis* or 11,13-di-*cis* isomer 7: $\lambda_{\text{max}}(\text{ethanol}) = 346$ nm, for both). Products detected in standards and aerosols are summarized in Table 2.

Doses were estimated based on the calculation, from the initial RA amount of the aerosol preparation subtracting the determined RA amount left in the PARI minus the RA amount trapped in the outlet filter (i.e., the amount of the RA aerosol that was not inhaled). Mean values for the determined concentrations of the RA leftover, the filter-trapped RA amount, and the calculated values for the estimated delivered doses are summarized in Table 3. Based on the initial average weight of 501.8 ± 8.5 g, the animals received daily an estimated average ATRA doses of $0.32 \text{ mg}\cdot\text{kg}^{-1}$ (low-dose group), $0.62 \text{ mg}\cdot\text{kg}^{-1}$ (medium-dose group), and $1.26 \text{ mg}\cdot\text{kg}^{-1}$ (high-dose group).

HPLC analysis of lung samples

Guinea pigs were subjected to a daily 20-min inhalation period for 6 days using one of three doses of RA (low, medium, high) or vehicle. To ensure an equal distribution of aerosol, each animal was repositioned each day. On the 6th day, guinea pigs were sacrificed 50, 100, or 150 minutes post treatment. Lung, liver, and plasma samples were extracted and analyzed for RA and isomers by HPLC (Figure 3). Controls had no detectable change in lung ATRA levels. The values for the low- and medium-dose groups almost doubled per 50-minute interval. However, the ATRA concentration in the high-dose group dropped to 25% of anticipated values at 150 minutes (Figure 4). Although ATRA accumulated in lung tissue, no dose-dependent effect was observed. Cellular lung levels of retinol, retinyl palmitate, and retinyl stearate appeared to be unaffected (245.6 ± 10.7 , 47.4 ± 3.4 , and 132.8 ± 7.7 ng/g wet weight, respectively). We found no accumulation of RA isomers in liver or plasma.

Western blots

In order to determine if inhaled ATRA was able to induce protein expression of CRBP-1, Western blot analyses were performed (Figure 5). Although some variation was observed between the room control (RC) and the inhalation vehicle control, CRBP-1 protein was

increased in a dose-dependent fashion at 100 minutes in the treatment compared to the control group (2-, 4, and 5-fold, respectively).

Discussion

ATRA was deposited in lung using this inhalation protocol, but not in plasma or liver. These results are in contrast to Taljanski et al. [22] who used a cyclosporin A/Cremophor aerosol in rats, which was absorbed by lungs but was also found in high concentrations in the systemic circulation.

The increase in lung ATRA levels at 100 minutes post inhalation (day 6) was similar for all three concentrations used. At 150 minutes, ATRA levels decreased in the high-dose group, whereas concentrations in the low- and medium-dose groups continued to rise. We believe the decrease at 150 minutes in the high-dose group reflects RA induction of enzymes that effectively metabolize/clear RA. In support of this, CYP26A protein levels were elevated and reached a plateau after 150 minutes in all 3 ATRA groups (data not shown). This could also explain why there was no clear dose-dependent effect on uptake.

Our future goal is to analyze and compare ATRA levels of mucous membranes of the trachea and the major bronchus with more peripheral parts of the lung in order to determine how far ATRA aerosol reaches into the lung.

Wang et al. [23] previously showed that ATRA induces cytochrome P450 3A4 (CYP3A4). At higher ATRA concentrations, CYP26 enzymes are likely to be saturated and CYP3A4 may play a more active role in RA clearance [24]. Based on these results, it is possible that an even lower concentration of the ATRA aerosol than used in these studies will be sufficient to induce genes of interest while limiting significant induction of clearance mechanisms.

There have been concerns raised about Cremophor EL if given intravenously, because of systemic effects include anaphylactic hypersensitivity, hyperlipidemia, and neurotoxicity [25]. However, rats exposed for over 8 hours to inhaled air saturated at 20°C with the volatile components of the product showed no irritation of their respiratory tracts [17]. In our studies, guinea pigs also had no visible signs of distress (normal behavior, no weight loss, no changes to fur, eyes or skin) during the course of the experiment.

We added BHT to enhance stability of the aerosol. Although BHT can produce lung damage and inflammation in mice [26], these effects have not been found in rats, hamsters, or guinea pigs [26,27]. Further large doses of BHT (>2000× the dose used here) were required to induce deleterious tissue effects in mice. Tocopherols were not considered as an alternative to BHT, since α -tocopherol can promote retinylester hydrolysis in rats [28,29].

HPLC analysis of our aerosol preparation of ATRA stored in -80°C for 16 months revealed that it was isomerized to 13-*cis*- and 9-*cis* isomers by only 3%. Only trace RA oxidation products were present (0.3%). ATRA standards always contain trace 13-*cis* isomer (0.55%) without detectable oxidization products.

Experiments with inhalable RA formulations have been reported by Dahl et al. [9], Wang et al. [10], and Raleigh et al. [30] using mice and rats. In those studies, nebulizing solutions consisted of either ethanolic solutions of 13-*cis* RA [9,10] or a polyethylene glycol 300/ethanol-based mixture, using a PARI LC Star nebulizer. In Raleigh et al. [30], aerosol formation was achieved with powdered 13-*cis* RA, dispersed with air. Brooks et al. [31] used a metered dose inhaler with hydrofluoroalkane 134a as propellant. We used a Cremophor-based nebulizing solution to ensure 100% miscibility of ATRA with water.

We chose to deliver ATRA as a bolus, as 13-*cis* RA failed to enhance lung or liver RAR β or lung RAR γ mRNA levels in retinol-deficient rats [32]. Further, a growing body of evidence indicates that 13-*cis* RA has several different functions than ATRA [33].

Massaro and Massaro [6] report in their work that RA treatment reverses anatomical characteristics and increases lung volume of elastase-induced emphysema in Sprague-Dawley rats. Although demonstrating a beneficial effect, animals received the RA treatment via intraperitoneal administration. In contrast, a study by March et al. [34] couldn't demonstrate any ATRA-associated reversal of lung damage associated with the emphysema model used comparing injection-treated and inhalation-treated Fischer 344 rats. Interestingly, a very recent case study [35] on a 45-year-old patient with heavy emphysema, never-smoker individual on a 6-month controlled ATRA inhalation therapy, showed signs of improvement in daily life and also in a variety of biochemical marker levels.

Recent work in our laboratory indicates that rodent species such as mouse and rat differ significantly in their lung vitamin A profile in comparison to guinea pig and human, which show a remarkable similarity [18]. This raises the question whether substantial changes also exist in the detailed regulation of the vitamin A signaling pathway and RA-dependent gene expression, which in turn would still leave the question open if inhaled ATRA can prevent, alter, or abrogate pathological conditions in lung cancer, chronic lung obstructive pulmonary disease (COPD), and emphysema.

Different forms of cancer are associated with disturbances in the RA signaling pathway, including unresponsiveness towards ATRA. RAR β has been identified as a tumor suppressor gene, which is silenced in certain forms of lung cancer [36]. The aberrant expression of the cellular RA-binding protein-2 (CRABP-2) is associated with RA resistance of tumor cells, since the main function of CRABP-2 is to channel ATRA directly into the nucleus [37]. In an effort to overcome ATRA resistance in non-small-cell lung carcinomas [38], A549 human lung cancer cells, resistant to the growth inhibitory effects of ATRA, have been incubated with ATRA incorporated in DOTAP (1,2 dioleoyl-3-trimethylammonium propane)/cholesterol liposomes, with a positive response to ATRA. This delivery system and formulation therefore may serve a helpful function in the treatment of cancers previously thought to be ATRA resistant, which warrants further investigations. Our preliminary results indicate that our water-soluble ATRA formulation is also capable of inhibiting cell growth in A549 cells (unpublished observation) and thus overcomes RA resistance often seen using other delivery methods.

We demonstrate here that Cremophor EL-based ATRA aerosol induced CRBP-1 expression in guinea pig lungs, indicating the delivery of solubilized ATRA to the nucleus. During the course of our experiments, we did not observe any toxic side effects or even tachyphylaxis during treatment with ATRA aerosol. Thus we have a suitable application at hand for a topical delivery of ATRA to guinea pig lungs.

Our Cremophor-based nebulizing solution could potentially be used to emulsify other related compounds, such as carotenoids and retinyl esters in order to study their topical applicability. Indeed, Biesalski et al. [39] demonstrated that inhalation of a retinyl palmitate aerosol can successfully improve the vitamin A status of preschool children. Furthermore, Kohlhäufel et al. [40] reported a complete reversal of 44% and partial remission in 12% of biopsies from patients with premalignant lesions of the bronchial epithelium.

However, because of the well-known side effects of retinoic acid, further detailed analyses on potential damage on cellular levels (by immunohistochemistry) are needed and to determine whether ATRA doses could be even reduced to rule out any damage on healthy lung tissue and to maintain a beneficial effect.

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FIGURE 1.

PARI-based inhalation chamber for guinea pigs, with mouths and noses covered by masks (outlets). To ensure a constant flow of aerosol, a slight negative pressure is applied to the chamber's outlet.

FIGURE 2.

Chromatograms at a wavelength of $\lambda = 350$ nm: (A) Mixture of retinoid standards; (B) all-*trans* RA nebulizing solution, fresh; (C) aerosol after 16 months of storage at -80°C ; (D) aerosol after 16 months of storage, 3–7-min time window for polar retinoids. Peak assignments: 1 = 13-*cis* RA; 2 = 9-*cis* RA; 3 = ATRA; 4 = all-*trans* retinol; 5, 6 = oxidized retinoic acid; 7 = di-*cis*-retinoic acid.

FIGURE 3.

(A) Chromatogram of control lung (inhalation of vehicle only) at $\lambda = 350$ nm. (B) Chromatogram of lung in mid-dose group at $\lambda = 350$ nm. Peak assignments: x = unknown; 1 = 13-*cis* RA; 3 = ATRA; 4 = all-*trans* retinol; 8 = all-*trans* retinyl palmitate; 9 = all-*trans* retinyl stearate; IS = all-*trans* retinyl acetate (internal standard).

FIGURE 4. Accumulation of ATRA in lungs from treated (low, medium, and high doses) and controls (C, vehicle) 50, 100, and 150 minutes following final inhalation, with 3 animals per group and 1 animal per time point; $n = 1$.

FIGURE 5.

(*Lower panel*) Western blot analyses of CRBP-1 levels in lung: RC (no treatment), C (control, vehicle only), and L (low-dose; 1.39 mM), M (medium-dose; 2.78 mM), and H (high-dose; 5.55 mM) ATRA groups, 100 minutes post final inhalation. (*Upper panel*) Histogram of the Western blots. Values normalized against β -actin. Samples from 1 animal per group per time point.

Aerosol Composition

TABLE 1

Composition	RA	Vehicle
All- <i>trans</i> retinoic acid	1.39, 2.78, or 5.55 mM	—
Cremophor EL	25%	25%
BHT	0.01%	0.01%
PBS (10×)	10%	10%
Water	Add to total volume	Add to total volume

TABLE 2

Isomers and Oxidation Products (%)

	Fresh aerosol preparation	Stored aerosol preparation ^a	Leftover in PARI	Aerosol in filter ^b	ATRA standard
4-oxo RA	0.23	0.12	0.12	0.28	n.d. ^c
5,6-epoxy RA	0.23	0.20	0.20	n.d.	n.d.
13- <i>cis</i> RA	0.15	3.00	1.41	3.38	0.55
di- <i>cis</i> RA	0.08	0.91	0.40	1.52	n.d.
9- <i>cis</i> RA	0.04	0.37	0.14	0.94	n.d.
ATRA	99.28	95.40	97.73	93.89	99.45

^a After 16 months of storage at -80°C.^b RA aerosol trapped in filter.^c n.d. = not detected.

TABLE 3
Concentrations and Estimated Doses Delivered to Lung Tissues

Groups	Aerosol (RA) (mM)	(mg/3 mL)	RA left in PARI (mg) *	RA trapped in filter (mg) *	Estimated dose per animal per inhalation (mg) *	% of total aerosol preparation
Control	0	0	0	0	0	0
Low dose	1.39	1.26	0.57 ± 0.05	0.22 ± 0.01	0.16 ± 0.02	12.49
Medium dose	2.78	2.49	1.10 ± 0.04	0.46 ± 0.03	0.31 ± 0.02	12.50
High dose	5.55	5.01	2.26 ± 0.10	0.87 ± 0.05	0.63 ± 0.05	12.52

* Given as mean values ± SEM, based on $n = 6$ determinations.