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ORIGINAL ARTICLE

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Improved respiratory delivery of the anticancer drugs, camptothecin and paclitaxel, with 5% CO_2-enriched air: pharmacokinetic studies

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Abstract *Purpose*: To increase pulmonary deposition of anticancer liposome aerosols in mice by modulation of respiratory physiology through the addition of 5% CO_2 to the air source used to generate the aerosols. Breathing CO_2-enriched aerosol increases pulmonary ventilation with concurrent increased deposition of inhaled particles. *Methods*: Dilauroylphosphatidylcholine liposome formulations of two anticancer drugs, paclitaxel (PTX) and camptothecin (CPT), were investigated. The aerosol droplet size was measured using an Andersen cascade impactor. Drug concentrations in aerosol droplet fractions and tissues were determined by HPLC analysis. ICR mice were exposed to each liposome aerosol for 30 min. For each drug, one group of mice inhaled the drug-liposome aerosol generated with a mixture of 5% CO_2 in air and another group inhaled the drug-liposome aerosols produced with normal air. Tissue distribution and pharmacokinetics were determined for both drug delivery systems. *Results*: Significantly higher concentrations of PTX and CPT were found in organs of mice exposed to 5% CO_2-air aerosols compared to organs of mice exposed to normal air aerosols. The highest concentrations of drug were detected in the lungs and were two- to fourfold higher with 5% CO_2-air aerosols than with aerosols generated with normal air. Higher concentrations were also detected in liver, spleen, kidneys, blood, and brain. *Conclusion*: 5% CO_2 enrichment of air increased respiratory tract deposition of inhaled aerosol particles containing PTX and CPT.

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Introduction

The anticancer drugs, paclitaxel (PTX) and different camptothecin (CPT) derivatives, are clinically active in the treatment of a variety of human tumors, including lung cancer [14, 20]. At present, these drugs are given systemically by oral or intravenous routes of administration. The development of toxic side effects is often a major limitation in such therapeutic regimens. We have previously reported successful treatment of several subcutaneous human cancer xenografts in nude mice [9] and in experimental murine pulmonary metastasis [11] using liposomal formulations of CPT and 9-nitrocamptothecin (9NC) administered by the aerosol route as an alternative method of therapy. Pharmacokinetic studies of CPT in mice have shown that inhalation of liposomal CPT produces substantial drug levels in the lungs and other organs, which clear rapidly after cessation of aerosol delivery [10]. In spite of these levels, aerosol delivery systems are generally only 15–20% efficient in drug deposition [23, 24] and increasing pulmonary deposition would be advantageous.

Increased efficiency of drug deposition in the respiratory tract by the inhalation route may be achieved in several ways: (1) by changing the concentration of drug in the formulation used for aerosolization [25], (2) by using more efficient types of nebulizers [24]; (3) by increasing the duration of treatment; or (4) by changing the breathing patterns [6]. CO_2 is a natural modulator of respiration. Increased concentration of CO_2 in inhaled air increases pulmonary ventilation as a result of a substantial increase in tidal volume, which depends on the CO_2 concentration in the inspired air [13, 18]. Thus utilization of CO_2 as a modulator of inhalation therapy might result in more effective pulmonary drug deposition by aerosol.

In the present study we investigated the deposition of two anticancer drugs, CPT and PTX, in the respiratory

tract of mice using a mixture of 5% CO_2 and air to generate aerosols. Since both drugs have very low solubility in water, they were encapsulated into liposomal formulations. Tissue distribution and pharmacokinetic studies were performed for PTX and CPT after aerosol delivery. The results of this study support the use of liposome aerosol technology as a method of noninvasive drug delivery to the respiratory tract for the treatment of lung cancer.

Materials and methods

Chemicals

PTX was obtained from Xechem (New Brunswick, N.J.). CPT was obtained from Sigma (St. Louis, Mo.) and 9NC from ChemWerth (Woodbridge, Conn.). Dilauroylphosphatidylcholine (DLPC) was purchased from Avanti Polar Lipids (Alabaster, Ala.). Dimethyl sulfoxide was purchased from Sigma and other organic solvents of HPLC grade were obtained from Fisher Scientific. Sterile water for irrigation came from Baxter Healthcare Corporation (Deerfield, Ill.).

Mice

ICR mice (7–8 weeks old) were obtained from Harlan-Sprague Dawley (Indianapolis, Ind.) and housed in standard cages with food and water provided ad libitum. Experiments were completed under the protocol approved by the institutional animal care and use committee.

Preparation of liposomes

Stock solutions of DLPC, PTX and CPT were prepared in *t*-butanol at 100, 10 and 1 mg/ml, respectively, using previously described methods [10]. Aliquots of PTX and DLPC were mixed at a weight ratio of 1:10. The CPT to DLPC weight ratio was 1:50. The drug-phospholipid mixture was then frozen in liquid nitrogen and lyophilized overnight to a dry powder. The formulations were stored sealed at –20°C. Before use the mixtures were reconstituted with sterile water for irrigation and vortexed until a homogeneous multilamellar liposomal suspension was obtained. The initial concentrations of CPT and PTX in suspension prior to nebulization were 0.5 mg/ml and 10 mg/ml, respectively. The size of liposomes before and after nebulization was determined using a Nicomp submicron particle sizer, model 370 (NICOMP, Santa Barbara, Calif.).

Aerosol particle size characteristics

The characteristics of aerosol particles containing liposomal encapsulated drugs were estimated using an Andersen/ACFM nonviable ambient particle sizing sampler (Andersen Instruments, Atlanta, Ga.) as previously described [25]. The concentration of drug in aerosols produced by air or gas mixtures flowing at 10 l/min through an AERO-MIST nebulizer was also measured by collecting samples for 3 min starting 1 min after aerosolization initiation. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated as described previously [24, 25] using KaleidaGraph 2.0 software (Synergy Software, Reading, Pa.).

Aerosol delivery

Treatment of mice with aerosol was performed as previously described [9, 10, 11]. Briefly, an AERO-MIST jet nebulizer (CIS-USA, Bedford, Mass.) was used to generate aerosol particles at the air flow rate of 10 l/min. Mice were placed in a sealed plastic cage ($23 \times 18 \times 13$ cm) and exposed to aerosol for 30 min. The aerosol was generated with normal or 5% CO_2-enriched air obtained by mixing normal air and CO_2 with a blender (Bird 3M, Palm Springs, Calif.) and the CO_2 concentrations were calibrated with a Fluid Fyrite (Bacharach, Pittsburgh, Pa.). At each time-point three mice were removed from the cage and killed by exposure to Isoflurane USP (Abbott Laboratories, Chicago, Ill.) and exsanguination. Organs were resected, weighed and kept frozen at –70°C until extraction.

Extraction of drug from tissues

Before extraction, samples were thawed and immediately cut into small pieces with scissors. To extract PTX from tissues, 3 ml ethyl acetate was added to each sample which was then homogenized in a mini-beadbeater (Wig-L-Bug, Model 3110B, Crescent Dental, Lyons, Ill.) for 2 min. Homogenates were transferred to 10 ml conical glass centrifuge tubes and centrifuged at 1000 *g* for 10 min. The supernatant fraction was separated and organic solvent was evaporated with air. The residue was reconstituted in 0.2 ml methanol/acetonitrile (2:1, v/v), sonicated in a waterbath sonicator and centrifuged at 1000 *g* for 10 min. Supernatant fractions were warmed at 37°C for 30 min and analyzed by HPLC.

The extraction procedure for CPT and 9NC has been described previously [10]. Briefly, after thawing, 20 μg 9NC in 20 μl was added to organs as an internal standard to determine the extraction efficiency. The samples were cut into small pieces and 1 ml 0.1% aqueous acetic acid solution, pH 3.2, was added to each sample. After homogenization in a mini-beadbeater, the homogenates were centrifuged at 1000 *g* for 5 min. The supernatant fractions were reextracted with 8 ml methylene chloride. The organic fraction was separated and dried under air at room temperature. The dried samples were reconstituted in 0.2 ml acetonitrile.

HPLC analysis

PTX was quantified by reverse-phase HPLC with monitoring on a Waters 486 UV absorbance detector at 227 nm (Waters, Milford, Mass.). All measurements were made at room temperature on a Waters Nova-Pak C18 column (3.9×150 mm). The mobile phase was composed of 49% acetonitrile and 51% water. The flow rate was 1.5 ml/min. A 25-μl aliquot of each sample was injected and data were analyzed with Waters Millennium Software. For PTX extraction efficiency determination, identical procedures were performed when a known amount of PTX was added to each tissue and compared with the extracted amount of PTX. The extraction efficiency (percent) was calculated as [(amount of PTX after extraction)/(amount of PTX added)] $\times$ 100. For all tested tissues the average extraction efficiency was $80 \pm 4\%$ (data not shown) and this index was used to calculate the final concentrations of drug in the tissues.

HPLC analysis of CPT was performed using a Waters Nova-Pak C18 column (3.9×150 cm) [10]. Chromatograms for CPT were monitored on a Waters 470 scanning fluorescent detector (λ_{ex} 360 nm, λ_{em} 455 nm) while 9NC was detected using a Waters 440 UV absorbance detector monitoring at 254 nm. The mobile phase was composed of 30% acetonitrile and 70% 0.1% acetic acid solution in water, pH 3.5, at a flow rate 1.2 ml/min [9, 10].

Results

Aerosol characteristics of liposome formulations

The properties of CPT-DLPC and PTX-DLPC liposomes and their aerosol characteristics are summarized

Table 1 Aerosol and liposome characteristics for PTX-DLPC and CPT-DLPC formulations using 5% CO_2-enriched air or normal air. Values are means ± SD, $n = 3$ for each value (*MMAD* mass median aerodynamic diameter, *GSD* geometric standard deviation)

Drug formulation	Air composition	Drug concentration in aerosol (μg/l)	Aerosol droplets		Liposome particle size (μm)	
			MMAD (μm)	GSD	Before nebulization	After nebulization
CPT-DLPC (0.5 mg CPT/ml)	Normal	9.0 ± 1.3	1.6 ± 0.3	2.1 ± 0.1	3.72 ± 1.10	0.34 ± 0.11
	5% CO_2	9.2 ± 1.9	1.7 ± 0.5	2.3 ± 0.2	2.54 ± 0.91	0.49 ± 0.07
PTX-DLPC (10 mg PTX/ml)	Normal	153 ± 27	2.0 ± 0.2	1.8 ± 0.03	12.49 ± 8.06	0.13 ± 0.18
	5% CO_2	175 ± 9	2.2 ± 0.2	1.9 ± 0.1	13.14 ± 12.15	0.23 ± 0.17

in Table 1. The utilization of 5% CO_2-enriched air did not change the concentration of either drug in the aerosol or their MMAD or GSD ($P > 0.1$, Student's *t*-test, two-tailed). As has been shown in our previous study [9], the nebulization procedure reduces the size of liposome particles from micron- to nano-particles, and this was found for both drug formulations in the present study. The size of liposomes of CPT-DLPC decreased from 2.54 ± 0.91 μm before nebulization to 0.49 ± 0.07 μm after nebulization using the 5% CO_2-air mixture. For the PTX-DLPC formulation these values were 13.14 ± 12.15 μm and 0.23 ± 0.17 μm, respectively. The liposome particle size before and after nebulization was not different for either PTX-DLPC or CPT-DLPC administered by aerosol using normal or 5% CO_2-enriched air ($P > 0.5$, Student's *t*-test, two-tailed).

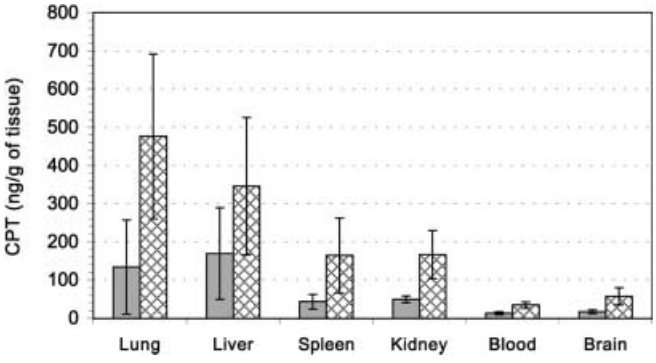

Fig. 1 Tissue distribution of CPT after a 30-min exposure to liposome aerosol generated with normal air (*solid gray*) or with 5% CO_2-enriched air (*hatched*). At the end of treatment (30 min) organs from three mice per group were resected and the drug content determined by HPLC. Mean values and SD were calculated. *P*-values for 5% CO_2-enriched air compared to normal air were 0.02, 0.13, 0.04, 0.04, 0.03, 0.01 for lungs, liver, spleen, kidney, blood and brain, respectively (Student's *t*-test, two-tailed)

Tissue distribution and pharmacokinetics of CPT-DLPC after delivery by aerosol generated with normal or 5% CO_2-enriched air

ICR mice were divided into two groups. The first group ($n = 4$) received the CPT-DLPC formulation via aerosol generated with normal air for 30 min, so their breathing parameters were not changed during treatment. The second group ($n = 6$) inhaled the same formulation but in an atmosphere of 5% CO_2-enriched air.

Inhalation of aerosols generated with 5% CO_2-enriched air resulted in a significant increase in deposition of CPT into the lungs (3.5-fold; Fig. 1). CPT was detected at 134 ± 123 and 476 ± 216 ng/g of lung tissue of mice of the first and second groups, respectively. The concentrations of drug in the liver, spleen, kidney, blood and brain after inhalation of CPT-DLPC aerosol generated with 5% CO_2-enriched air were also increased.

In additional experiments, the pharmacokinetic deposition of CPT in lungs during and after 30 min exposure to aerosols of CPT-DLPC using normal or 5% CO_2-enriched air was determined (Fig. 2). The pulmonary concentrations of CPT increased during treatment with the maximum concentration (C_{max}) at the end of aerosol treatment (30 min). The peak respiratory levels were 232 ± 158 and 486 ± 78 ng/g of tissue for normal and 5% CO_2-enriched air, respectively. During the 15 min after aerosol treatment had been stopped, the concentrations of the drug decreased exponentially. Clearance half-lives ($T_{1/2}$) for both treatments were 12–15 min. The profiles of the pharmacokinetic curves were very similar for both treatments. There were only trace amounts of drug detected in the lungs 90 min after the end of aerosolization (120-min time-point) with both air sources.

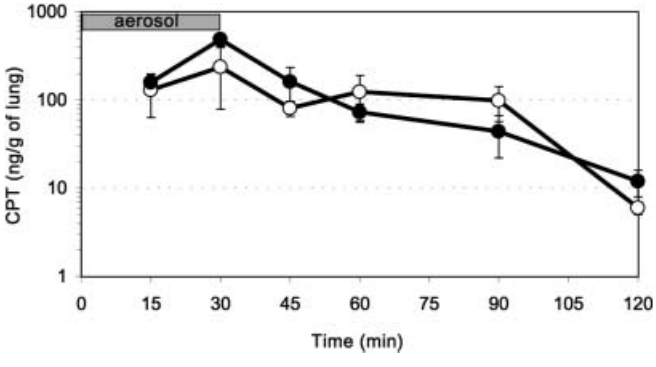

Fig. 2 Pulmonary concentration-time curve for CPT liposomes administered for 30 min by aerosol generated with normal air (○) or with 5% CO_2-enriched air (●). For each time-point lungs from three mice were resected and the drug content determined by HPLC. Mean values and SD were calculated

Tissue distribution and pharmacokinetics of PTX after treatment with PTX-DLPC aerosol generated by normal or 5% CO_2-enriched air

Because of limitations of the detection method, we used a PTX liposome formulation at 10 mg PTX/ml suspension for these studies. Mice were killed halfway through exposure (15 min), at the end of treatment (30 min), and at several time-points following the end of treatment. Mice were exposed to PTX-DLPC aerosol generated with either normal air or 5% CO_2-enriched air.

Pulmonary PTX C_{max} values were achieved at the end of treatment (30 min) with both air sources (Fig. 3). In the 5% CO_2-enriched air group, C_{max} was 4.2-fold higher than in the ambient air group (23.1 ± 4.3 and 5.5 ± 0.2 μg/g, respectively). The use of 5% CO_2-enriched air produced a 5.7-fold higher area under the lung concentration-time curve compared to the use of normal air (33.7 and 5.9 μg·h/g, respectively). In both cases PTX concentrations in the pulmonary tissue started to decrease after the treatment ended. $T_{1/2\alpha}$ and $T_{1/2\beta}$ values for PTX in the lungs were 0.3 and 1.6 h, respectively, when normal air was used for aerosol generation, and 0.7 and 5.1 h, respectively, when 5% CO_2-enriched air was used for aerosol generation. Comparative analysis for the other organs including liver, spleen, kidney and blood were done, but the levels of PTX in these tissues using normal air for aerosolization were below detectable levels.

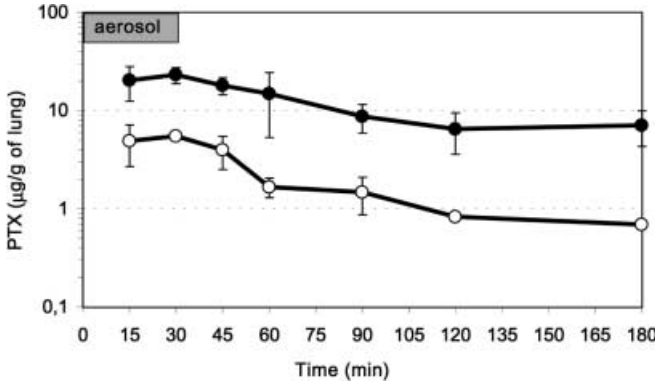

Fig. 3 Pulmonary concentration-time curve for PTX liposomes administered for 30 min by aerosol generated with normal air (○) or with 5% CO_2-enriched air (●). For each time-point lungs from three mice were combined and the drug content determined by HPLC. Each experiment was repeated three times and mean values and SD were calculated

The tissue distribution of PTX after liposome aerosol delivery using 5% CO_2-enriched air is presented in Table 2. The highest concentrations of the drug were detected in the lungs. Lower concentrations were found in the other organs. Analysis of the area under the concentration-time curve (AUC) over a 3-h period for different organs using the trapezoidal rule showed the following AUC values for lungs, liver, spleen, kidney, blood and brain: 34 ± 2, 9.8 ± 1.9, 2.4 ± 1.5, 2.8 ± 1.5, 0.13 ± 0.10, 0.23 ± 0.2 μg PTX·h/g tissue, respectively.

Discussion

CPT analogues and taxanes are among the novel promising agents being developed for lung cancer chemotherapy. These drugs have shown beneficial results in clinical trials when used as single agents or in combination with other drugs [14]. The effective routes and vehicles for drug administration have been studied extensively for both of these water-insoluble drugs. Several studies have revealed that liposomes can be used as effective and safe vehicles for these lipophilic drugs [1, 2, 3, 16]. To date, the most effective route for PTX administration has been continuous intravenous infusion [15, 17]. For lipophilic congeners of CPT, oral administration has been the most effective in human trials [19, 22].

Using these systemic routes of drug delivery, a certain amount of drug will egress from the blood stream and localize in the respiratory tissue, but lungs are not the main organs for drug deposition. The utilization of conventional liposomes as carriers for these drugs does not improve the pulmonary deposition of drugs administered by commonly used systemic routes [2, 21]. In our previous experiments with CPT, it was shown that nebulization is a very effective route for target drug delivery to the respiratory tract [10]. Promising results have been obtained when the aerosol route was used with new formulations of doxorubicin and PTX to treat dogs with spontaneously arising primary and metastatic lung tumors [8]. In all these previous studies normal air was used to generate aerosols. In the present study we demonstrated improved deposition of CPT and PTX liposomes using CO_2-enriched air for aerosolization. The peak concentrations of CPT in the lungs of ICR mice were enhanced up to 2.1–3.5-fold when 5% CO_2-enriched air was used for aerosolization than when normal air was used. For the PTX formulation the inhalation of 5% CO_2-enriched air increased pulmonary

Table 2 PTX deposition in tissues during and after 30 min exposure to aerosol PTX-DLPC generated with 5% CO_2-enriched air. Values are mean ± SD PTX concentrations (μg/g of tissue) from three experiments (organs from three mice were combined and processed in each experiment)

Time (h)	Lungs	Liver	Spleen	Kidney	Blood	Brain
0.25	20.3 ± 7.8	1.5 ± 0.8	0.6 ± 0.3	1.4 ± 0.0	0.25 ± 0.03	0.14 ± 0.16
0.5	23.1 ± 4.3	5.7 ± 3.0	1.4 ± 0.9	1.6 ± 0.1	0.18 ± 0.08	0.16 ± 0.02
0.75	18.0 ± 3.6	5.5 ± 1.8	0.5 ± 0.4	1.4 ± 0.1	0.08 ± 0.09	0.11 ± 0.03
1.0	14.8 ± 9.5	4.8 ± 3.9	2.6 ± 2.7	1.2 ± 0.7	0.07 ± 0.07	0.11 ± 0.03
1.5	8.7 ± 2.8	2.8 ± 0.8	1.0 ± 1.6	0.7 ± 0.3	0.03 ± 0.06	0.09 ± 0.08
2.0	6.5 ± 2.9	3.1 ± 0.7	0.6 ± 0.4	0.4 ± 0.3	0.01 ± 0.02	0.04 ± 0.04
3.0	7.1 ± 2.8	2.3 ± 0.6	0.5 ± 0.2	0.4 ± 0.1	0.01 ± 0.02	0.05 ± 0.05

peak concentration of drug more than 4.2-fold compared to the inhalation of normal air.

The increased pulmonary drug concentrations found in the lungs after inhalation of 5% CO_2-enriched air could be explained by changed respiratory patterns. We visually observed that the breathing patterns of mice in the atmosphere of 5% CO_2-enriched air became deeper and slower. The breathing patterns returned to normal almost immediately after the end of treatment. Histological analysis did not reveal any changes in pulmonary tissue [5]. Plethysmograph studies performed by other researchers have revealed that inhalation of 5% CO_2-enriched air increases ventilation in mammals primarily because of the increase in tidal volume (approximately 170–180%) [12, 13]. In our studies with these two different drugs the average pulmonary deposition increased approximately two- to fourfold. This disproportion with the increase in tidal volume may be due to some other physiological changes in breathing parameters, such as breathing frequency, duration of inspiratory and expiratory cycles, and minute ventilation volume [4]. It has been shown that by deep and complete expiration with breath-holding the retention of the aerosol increases almost twice in comparison with normal breathing [7].

The use of 5% CO_2-enriched air did not change tissue distribution patterns in the studies with CPT. We observed increased drug accumulation also in other organs, such as the liver, spleen, kidney, blood and brain. The profiles of drug pharmacokinetic curves looked very similar for both air sources. Peak concentrations for both drugs were observed at the end of aerosolization after which lung concentrations started to decline.

The results of this study demonstrate the feasibility for improved pulmonary delivery of chemotherapeutic agents using CO_2-enriched air for nebulization. In studies with humans, it has been shown that the inhalation of air containing low concentrations of CO_2 (3–7%) causes similar changes in breathing patterns and is tolerated well [4, 18]. No difference in breathing pattern has been observed between inhalation 5% CO_2-enriched air and moderate physical exercise in humans [26]. We believe that similar effects of 5% CO_2-enriched air may be obtained in humans using the aerosol treatment described here.

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